Docket No.: 377882000800 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Gary VAN NEST et al.

Application No.: 09/642,492 Confirmation No.: 7136

Filed: August 18, 2000 Art Unit: 1648

For: METHODS OF MODULATING AN IMMUNE RESPONSE USING IMMUNOSTIMULATORY SEQUENCES AND COMPOSITIONS FOR USE THEREIN Examiner: E. Le

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is an Appeal from the final rejection mailed June 13, 2005 and the Advisory Action mailed January 26, 2006, finally rejecting claims 1, 13-23, 25-33, and 37-52 under 35 U.S.C. §§ 112 ¶1 and 103. A Notice of Appeal was filed on December 12, 2005. This brief is filed within seven months of filing the Notice of Appeal, and is in furtherance of said Notice of Appeal. In accordance with MPEP § 1205.01, the two-month period of time set by 37 C.F.R. § 41.37(a) for filing the Appeal Brief after the Notice of Appeal may be extended by up to five months. Appellants submit herewith a petition and fee for a five-month extension of time under 37 C.F.R. § 1.136(a), thereby extending the deadline for filing the Appeal Brief to July 12, 2006. Accordingly, this Appeal Brief is timely filed.

The fees required under 37 C.F.R. § 41.20(b)(2) are dealt with in the accompanying Fee Transmittal.

This brief contains items under the following headings as required by 37 C.F.R. § 41.37 and M.P.E.P. § 1206:

I. Real Party In Interest
II Related Appeals and Interferences
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I. REAL PARTY IN INTEREST

The real party in interest for this appeal is the assignee of record, Dynavax Technologies Corporation, 2929 Seventh Street, Suite 100, Berkeley, CA 94710.

II. RELATED APPEALS, INTERFERENCES, AND JUDICIAL PROCEEDINGS

There are no other pending appeals, interferences, or judicial proceedings known to Appellant, Appellant's undersigned attorney, or assignee which will directly affect or be directly affected by, or have a bearing on, a decision by the Board of Patent Appeals and Interferences in the presently pending appeal.

III. STATUS OF CLAIMS

There are 37 claims pending in this application. Claims 1, 13-23, 25-33, and 37-42 are on appeal. Claims 2-12, 24, 34-36, and 53 were canceled. Claims 43-52 were withdrawn from consideration but not canceled. Claims 1, 13-23, 25-33, and 37-52 are pending. No claims stand allowed. Claims 1, 13-23, 25-33, 25-33, and 37-42 stand rejected.

IV. STATUS OF AMENDMENTS

Applicant filed a Response After Final Rejection and a Notice of Appeal on December 12, 2005. No claim amendments were made in the Response After Final Rejection. The Examiner responded to the Response After Final Rejection with an Advisory Action mailed January 26, 2006. In the Advisory Action, the Examiner indicated that Applicants' response would not be sufficient to place the claims in condition for allowance. No amendments were filed subsequent to the final rejection. Thus, the claims that appear in Appendix A are the claims as pending.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The claimed invention is based on the discovery that modulation of an immune response to an antigen (a "second antigen") may be achieved by co-administration of the antigen and another antigen (a "first antigen") that is covalently conjugated to an immunomodulatory polynucleotide that contains an immunostimulatory sequence ("ISS"). Administration of the immunomodulatory polynucleotide-first antigen conjugate elicits an immune response, particularly a Th1 response, to the second antigen.

ISS polynucleotides were well known in the art prior to filing of this application, as well as how to make them and how to identify and test such sequences for immunostimulatory activity. It was also known in the art that conjugation of an antigen to an ISS will stimulate an immune response. However, it was not known until the present invention by Applicants that administration of an ISS-antigen conjugate could stimulate an immune response to a second, unconjugated antigen.

The inventors have discovered that administration of an ISS-containing polynucleotide–first antigen conjugate advantageously permits immune modulation at a significantly lower dose and with a stronger interferon gamma response and an enhanced CTL response with respect to the second antigen, as compared to administration of second antigen alone.

The benefit of this novel approach is that it alleviates the need to design and manufacture different formulations for each antigen against which generation of an immune response would be desirable. For example, in the context of immunization against a pathogen, rapid mutation in some antigenic proteins, such as coat proteins, would not necessitate characterization of the changes and reformulation of a vaccine to reflect those changes, because administration of an immunomodulatory polynucleotide-first antigen conjugate enables immunization against antigenic determinants of the co-administered pathogen regardless of the polypeptide sequences of the antigenic portions.

Claims under consideration

There are three independent claims under consideration in the application on appeal, claims 1, 37, and 40.

Claim 1

Claim 1 is directed to a method of modulating an immune response to a second antigen in an individual comprising co-administering (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen, and (ii) a second antigen, at the same site in the individual and in an amount sufficient to modulate an immune response in the individual to the second antigen, wherein the polynucleotide comprises an immunostimulatory sequence ("ISS") comprising the sequence 5'-cytosine, guanine-3'.

Dependent claims add limitations that further specify the nature of the first antigen, further specify that the immune response is modulated by stimulating a Th1 response to second antigen and specify stimulation of production of specific molecules associated with the immune

response, further specify sequence requirements for the ISS, or further specify that the individual is a mammal, such as a human.

Claim 37

Claim 37 is directed to a composition comprising (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen, and (ii) a second antigen, wherein the polynucleotide comprises an ISS comprising the sequence 5'-cytosine, guanine-3', and wherein the first antigen is a viral conserved polypeptide and the second antigen is a viral variable polypeptide.

Dependent claims add limitations that further specify the nature of the first antigen.

Claim 40

Claim 40 is directed to a composition comprising (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen, and (ii) a second antigen, wherein the polynucleotide comprises an ISS comprising the sequence 5'-cytosine, guanine-3', and wherein the first antigen is an allergen .

Dependent claims add limitations that further specify the nature of the first antigen.

Support and significance of claim terms

The support in the specification and significance of each of the features recited in the independent claims is discussed below:

Modulating an immune response "Modulating an immune response" refers to immunostimulatory and immunosuppressive effects, as described on page 9, line 31 – page 10, line 15.

Individual The term "individual" refers to a vertebrate, preferably a mammal, such as a human, as described on page 13, lines 1-3 of the specification.

Co-administering The term "co-administering" refers to the administration of at least two different substances, *i.e.*, an immunomodulatory polynucleotide-first antigen complex and a second antigen, sufficiently close in time to modulate an immune response, as described on page 13, lines 12-15.

Complex The term "complex" in the context of a conjugate as claimed, refers to a linked ISS-containing polynucleotide and antigen, as described on page 10, lines 16-18.

Immunomodulatory polynucleotide An "immunomodulatory" polynucleotide refers to a polynucleotide that mediates immunostimulatory or immunosuppressive effects, as described on page 9, line 31 – page 10, line 15. A "polynucleotide" or "oligonucleotide" is described on page 9, lines 25-30, as including single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides or combinations thereof. The oligonucleotide can be linearly or circularly configured, or the oligonucleotide can contain both linear and circular segments. (The terms "polynucleotide" and "oligonucleotide" are used interchangeably in the specification.)

Covalently conjugated A covalent "conjugate" refers to a complex in which an ISS-containing polynucleotide and an antigen, *i.e.*, the "first antigen" in the claimed methods and compositions, are covalently linked, as described on page 10, lines 16-18.

First antigen The term "first antigen," *i.e.*, the antigen that is covalently conjugated to the immunomodulatory polynucleotide in the claimed methods and compositions, refers to a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor, as described on page 10, lines 19-26.

Second antigen The term "second antigen" refers to a different antigen than the first antigen, including a different antigenic region within the same polypeptide, which is administered to an individual, and against which an immune response is modulated by the methods of the invention, as described on page 10, lines 27-32.

At the same site The specification describes administration of the first antigen and immunomodulatory polynucleotide at the same site as the second antigen on page 15, lines 19-23. The specification states that "[f]or example, if the second antigen is encountered at the mucosa, such as lung or vaginal tissue, then the immunomodulatory polynucleotide and first antigen are administered to the relevant mucosa." In the context of the claimed methods, in which the immunomodulatory polynucleotide-first antigen complex is co-administered with the second antigen at the same site, both components are administered to the same tissue site sufficiently close in time to modulate an immune response (see description of "co-administration" on page 13, line 12-15).

Amount sufficient to modulate an immune response A "sufficient amount" to modulate an immune response refers to the amount of the claimed immunomodulatory polynucleotide-first antigen complex that is sufficient to effect beneficial or desired results, *i.e.*, an amount sufficient to modulate an immune to a second antigen compared to the immune response obtained when the second antigen is administered alone, as described on page 13, lines 4-11.

Immunostimulatory sequence ("ISS") The terms "immunostimulatory sequence" and "ISS" refer to a polynucleotide sequence that effects a measurable immune response as measured *in vitro*, *in vivo*, and/or *ex vivo*, as described on page 9, lines 17-24.

Comprising the sequence 5'-cytosine, guanine-3' The ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine, guanine-3', as described on page 16, lines 11-12. Other embodiments of the ISS sequence are described on page 16, line 13 – page 17, line 26.

Viral conserved polypeptide A viral "conserved" polypeptide refers to a viral polypeptide (or a region or domain of a polypeptide) that does not mutate, or change its sequence, at an appreciable rate, for example, a constant domain that is not prone to vary between strains and/or species of virus, as described on page 11, lines 13-25.

Viral variable polypeptide A viral "variable" polypeptide refers to a viral polypeptide (or a region or domain of a polypeptide) that mutates at an appreciable rate, for example, some viral coat proteins, as described on page 11, lines 25-31.

Allergen The term "allergen" refers to an antigen or antigenic portion of a molecule, usually a protein, which elicits an allergic response upon exposure to a subject, as described on page 12, lines 21-27. Examples of a number of isolated allergens are provided in Table 1 on pages 22-25.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The grounds of rejection on Appeal are:

(1) whether one of skill in the art would be able to practice the invention of claims 1, 13-23, 25-30, 32, 33, and 37-42 without undue experimentation, in accordance with the enablement requirement of 35 U.S.C. §112, first paragraph.

(2) whether claims 1, 13, 14, 17, 20-23, 25-33, 37, and 40-42 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997).

(3) whether claims 15 and 38 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Lee et al. (*Ann. Med.* 30:460-468, 1998).

(4) whether claims 16 and 39 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Durali et al. (*Journal of Virology* 72(5): 3547-3553, 1998).

(5) whether claims 18 and 19 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Anderson (U.S. Patent No. 4,673,574).

VII. ARGUMENT

A. The specification provides adequate guidance to enable claims 1, 13-23, 25-30, 32, 33, and 37-42, in accordance with 35 U.S.C. §112, first paragraph.

With respect to this rejection, claims 1, 13-23, 25-30, 32, 33, and 37-42 stand or fall together.

The Examiner alleges that claims 1, 13-23, 25-30, 32, 33, and 37-42 are not enabled by the specification. The Examiner concedes that the "specification . . . [is] enabling for using an ISS molecule comprising SEQ ID NO:1," but the Examiner contends that "the specification . . . does not reasonably provide enablement for IS sequences that are shorter or do not conform to the enabled motif." 8/18/00 Office Action, page 2. The basis of the Examiner's rejection is that "the scope of immunostimulatory sequences claimed would require an undue quantity of experimentation for one skilled in the art." 8/18/00 Office Action, page 5. The Examiner cites a reference by Fearon et al. (2003) *J. Immunol.* 33:2114-2122, as allegedly teaching that "the determination of whether [an ISS molecule] possesses immunomodulatory effects cannot be determined without experimentation" and "whether the genus of molecules encompassed by claims 1, 25-30 and 37 are immunogenic is unpredictable." 9/22/04 Office Action, pages 4-5. Applicants respectfully disagree.

1. The Examiner has not established a prima facie case for lack of enablement.

"To be enabling, the specification of a patent must teach those skilled in the art to make and use the full scope of the claimed invention without 'undue experimentation' . . . Nothing more than objective enablement is required, and therefore it is irrelevant whether this teaching is provided through broad terminology or illustrative examples." *See In re Wright*, 999 F.2d 1557, 1561 (Fed. Cir. 1993). With respect to the enablement requirement for patentability, the burden is on the Examiner to show that the specification is not enabling. MPEP § 2164.04 states that "[a] specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement

of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." The MPEP cites the decision in *In re Marzocchi*, 439 F.2d 220, 224 (CCPA 1971), in which the court stated that the Patent Office, when making a rejection on the basis of nonenablement, must explain why it doubts the truth or accuracy of the disclosure by backing up its assertion with acceptable contrary evidence or reasoning.

The Examiner has failed to meet the burden of showing that the specification does not provide an enabling disclosure. Applicants respectfully submit that the specification provides all the information required for one of skill in the art to make and use the invention to modulate the immune response to the second antigen as claimed.

a. The specification teaches how to make the claimed ISS-containing polynucleotides and covalent conjugates with a first antigen thereof.

The specification teaches the requirements for the ISS and the claimed ISS-containing immunomodulatory polynucleotide, and provides methods by which ISS can be made and evaluated for immunomodulatory activity. See, for example, page 15, line 30, to page 21, line 17. The specification states that "ISS have been described in the art and *may be readily identified using standard assays* which indicate various aspects of the immune response, such as cytokine secretion, antibody production, NK cell activation and T cell proliferation." Page 16, lines 3-5, emphasis added. The specification provides a number of references that describe ISS, methods for identifying ISS, and methods for evaluating immunomodulatory activity of ISS. (*See* page 3, line 3 – page 5, line 26, and page 16, lines 6-10.)

The specification discloses sequence requirements for the ISS of the claimed immunomodulatory polynucleotide and provides specific examples of ISS sequences. On page 16, lines 11-18, the specification teaches that "[t]he ISS can be of any length greater than 6 bases or base pairs and generally comprises the sequence 5'-cytosine,guanine-3', more particularly comprises the sequence 5'-purine,purine,C,G,pyrimidine,pyrimidine-3' (such as 5'AACGTT-3'), preferably greater than15 bases or base pairs, more preferably greater than 20 bases or base pairs in length. An ISS may also comprises the sequence 5'-purine,purine,C,G,pyrimidine,pyrimidine,C,G-

3'. . . .[A]n ISS may also comprise 5'-T,C,G-3'." The specification at page 16, line 19 – page 17, line 4 goes on to disclose 40 specific embodiments of sequences that the ISS may comprise.

The specification teaches that "[t]he ISS *can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art* including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel et al. (1987); and Sambrook et al. (1989)." Page 17, lines 27-30, emphasis added. Synthesis of an ISS would be routine in the art.

The specification also describes antigens and how to make covalently conjugated polynucleotide-antigen complexes for use in the invention. Production of a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen, as claimed, may be achieved using techniques that are routine in the art. Examples of antigens are described extensively on page 21, line 20 – page 27, line 30. Methods for covalently conjugating an antigen to an immunomodulatory polynucleotide are described on page 29, line 32 – page 31, line 5 and page 31, line 25 – page 32, line 19. The techniques that are described for effecting a covalent linkage are standard methods in the art, for example, linking of the polynucleotide to a peptide antigen containing an amino acid with a reactive group, for example, an N-hydroxysuccinimide ester linkage with the N^4 amino group of a cytosine residue (page 29, line 31 – page 30, line 4), linking at the 3' end of the polynucleotide to peptide via solid support chemistry (page 30, lines 9-25), or attachment at the 5' end of the polynucleotide via an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis (page 30, line 26 – page 31, line 5). Standard methodologies are also described for linkage of the polynucleotide to a lipid (page 31, lines 25-31), or to an oligosaccharide (page 32, lines 1-5).

In summary, the specification teaches sequence requirements for ISS and specific examples of ISS, and provides information regarding how to identify and evaluate other ISS using techniques that are well known in the art. Synthesis of ISS and covalent conjugation to an antigen may also be achieved using techniques that are described in the specification and are standard in the art. Thus, the specification provides adequate guidance regarding how to make ISS and the claimed complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen.

b. The specification teaches how to use the claimed immunomodulatory polynucleotide-first antigen complexes.

The specification provides guidance regarding administration of the claimed compositions. For example, suitable formulations and routes for administration are disclosed on page 42, line 9 – page 47, line 8. Methods to assess the modulation of the immune response to a second antigen as claimed are described on page 47. line 10 – page 49, line 5. Analysis of the immune response to the second antigen may be assessed, for example, by "measuring antigen-specific antibody production, activation of specific populations of lymphocytes such as $CD4^+$ T cells or NK cells, and or production of cytokines such as IFN, IL-2, IL-4, or IL-12." Page 47, lines 13-15. The specification teaches that standard methods in the art may be used to perform such measurements, such as enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS), or cytotoxicity assays. (See page 47, lines 15-24).

In addition, the specification provides a working example. Although working examples are not required for enablement (MPEP §2164.02), the claimed invention is exemplified in Example 1 provided on pages 49-56 of the specification. This example demonstrates that co-administration of an ISS-first antigen conjugate and a second antigen at the same site enhances the Th1 immune response to a second antigen, *i.e.*, modulates an immune response to a second antigen, as discovered and claimed by the inventors.

c. It would not require undue experimentation to make and use the claimed invention.

As discussed in detail above, the specification provides guidance regarding *how to make* ISS and covalently conjugated immunomodulatory polynucleotide-first antigen complexes as claimed. Such techniques are standard in the art. Moreover, sequence requirements for ISS are set forth in the specification, as well as 40 examples of specific ISS polynucleotide sequences, and methods for identifying and testing additional ISS are described in the specification and are well known and available in the scientific literature. Methods for *how to use* to the claimed compositions and for practicing the claimed methods are described in detail in the specification, in terms of formulations and routes of administration, as well as testing for modulation of an immune response,

using standard techniques in the art. In addition, a working example is provided showing that an ISS-first antigen conjugate modulates an immune response to a second antigen, as claimed.

For a *prima facie* case of non-enablement, the burden is on the Office to demonstrate that there is a reasonable basis to question, the presumptively sufficient disclosure made by the applicant. *See*, for example, *In re Wright*, 999 F.2d 1557 (Fed. Cir. 1993). Applicants respectfully submit that the Examiner has not produced adequate evidence to support a lack of enablement, *i.e.*, to establish that with the teachings provided in the specification, a person skilled in the art could not determine an ISS-containing immunomodulatory polynucleotide comprising a CG sequence that, when covalently conjugated to a first antigen, can modulate an immune response to a co-administered second antigen.

"The test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 785 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). Applicants submit that in the instant case, enablement is provided by the disclosure in the specification, and also by knowledge in the art about ISS polynucleotides. The Examiner states that "the specification, while being enabling for using an ISS molecule comprising SEQ ID NO: 1, does not reasonably provide enablement for IS sequences that are shorter or do not conform to the enabled motif." 6/13/05 Office Action, page 2. Applicants disagree with this statement by the Examiner. In addition to the guidance provided by the specification, described in detail above, immunostimulatory polynucleotides are well known in the art and polynucleotides with immunostimulatory sequences active in cells of many mammalian species have been described in the scientific literature, including humans, monkeys, chimpanzees, cows, swine, dogs, cats, rabbits, mice, and rats. In particular, much has been described about ISS activity in human cells and immunostimulatory sequences active in human cells have been the subject of much scientific and patent literature. Thus, Applicants submit that the ISS art is more mature than the Examiner suggests, and it would not require undue experimentation to apply the foundation provided by the ISS art to identify additional ISS sequences that will be useful in the practice of the claimed invention.

The court in found that the enablement requirement was satisfied by a "disclosure [that] provides considerable direction and guidance on how to practice [the] invention and presents working examples," in view of the fact that "[t]here was a high level of skill in the art at the time when the application was filed, and all of the methods needed to practice the invention were well known." *In re Wands*, 858 F.2d 731, 740 (Fed. Cir. 1988). The court in *United States v. Telectronics.* held that "[s]ince one embodiment [was] . . . disclosed in the specification, along with the general manner in which its current range was ascertained, . . . other permutations of the invention could be practiced by those skilled in the art without undue experimentation." *United States v. Telectronics, Inc.*, 857 F.2d 778, 786 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989). The Federal Circuit has stated that "[e]nablement is not precluded by the necessity for some experimentation such as routine screening." *In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988). Applicants respectfully submit that the specification provides a reasonable amount of guidance to the skilled artisan with respect to the direction in which the experimentation should proceed to optimize the teachings of the specification and the art and that any additional necessary experimentation is well within the level of ordinary skill in the art, *i.e.*, no *undue* experimentation is required. Applicants respectfully submit that varying the nucleic acid sequence of oligonucleotides and testing the oligonucleotides for immunostimulatory activity are well within the bounds of routine experimentation by one of skill in the art.

Therefore, given the guidance in the specification and in view of the working example demonstrating that the invention works as claimed, it would not require undue experimentation for a skilled artisan to practice the claimed invention. Applicants respectfully submit that the pending claims are in compliance with the enablement requirement and that the Examiner has not established a *prima facie* case for lack of enablement.

2. The Examiner's reliance on Fearon et al. is misplaced, and the teachings of this reference do not render the claimed invention non-enabled.

The Examiner has cited Fearon et al. (2003) *J. Immunol.* 33:2114-2122, as allegedly exemplifying the "unpredictable nature" of ISS molecules. 6/13/05 Office Action, page 3. The Examiner points to a single polynucleotide sequence in this reference that was shown to be non-

immunogenic, in the context of a polynucleotide non-covalently adsorbed to the surface of a polymeric microsphere, as allegedly showing that "[t]he skilled artisan would be unable to predict which characteristics are required for a sequence to be immunostimulatory since ACGTTCG of Fearon et al. has two CpG motifs and is not immunogenic." 6/13/05 Office Action, page 3. The Examiner further states that "whether the genus of ISS molecules encompassed by claims 1, 25-30 and 37 is immunogenic is unpredictable and would require an undue quantity of experimentation to determine which species within the broad genus is immunogenic, since the presence of a CpG motif does not automatically equate to immunogenicity." 6/13/05 Office Action, sentence bridging bottom of page 3 and top of page 4.

a. A single alleged inoperative embodiment does not render the claimed invention non-enabled.

As is well established in patent law, enablement "is not precluded even if some experimentation is necessary, although the amount of experimentation needed must not be unduly extensive." *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986). Further, fulfillment of the enablement requirement does not require that every embodiment of the invention be predictable. Rather, unpredictability is permitted, the level of unpredictability permitted depending on the level of guidance provided by the specification and the knowledge in the art. Applicants note that the test for enablement is not whether a certain amount of experimentation is required to practice the claimed invention, but rather whether the amount of experimentation is "*undue*." *In re Wands,* 858 F.2d 731 (Fed. Cir. 1986). As discussed above, a great deal of guidance is provided by both the specification and the knowledge in the art. Applicants submit that with respect to identification and testing of polynucleotide sequences with immunostimulatory activity, the skilled artisan would be able to extend the teachings of the specification and the art to other immunostimulatory polynucleotides as claimed.

With respect to Fearon et al., the Examiner points to the inactivity of the sequence ACGTTCG in support of the enablement rejection and concludes that it would require undue experimentation to make and use the claimed invention commensurate with the scope of the claims, based on the teaching by Fearon et al. of a *single inoperative species*. However, the Examiner fails

to note that Fearon et al. demonstrate many other CG containing oligonucleotides of varying length and sequence with immunostimulatory activity. See, for example, Figs. 2 and 3 of Fearon et al.

Further, Applicants submit that the possibility that the invention may not work in every species encompassed by a claim does not necessarily render the claim nonenabled, because a claim may encompass inoperative embodiments. MPEP § 2164.08(b). In *Atlas Powder Co. v. DuPont*, 750 F.2d 1569, 1576 (Fed. Cir. 1984), the court stated that "[i]t is not a function of the claims to specifically exclude . . . possible inoperative substances." Further, the MPEP states that "[t]he standard [for enablement] is whether a skilled person could determine which embodiments. . . would be inoperative or operative *with the expenditure of no more effort than is normally required in the art.*" MPEP 2164.08(b), emphasis added. In *Atlas Powder* forty percent of about 300 experiments performed by the appellee failed for one reason or another. However, the Federal Circuit upheld the lower court's finding that the experiments were designated as failures because they were not optimal under all conditions, and held that optimality is not required because one skilled in the art would know how to modify those failures to achieve a better result. Applicants have invented a general method for modulating an immune response to a second antigen by co-administration with a first antigen conjugated to a polynucleotide comprising an ISS. One skilled in the art knows how to optimize the ISS sequence to achieve better results.

Further, in *In re Wands*, the court held that the enablement requirement was satisfied even though only 4 of 9 antibodies analyzed (44%) were found to have the claimed binding requirements and those successful 4 were produced in only 2 of 10 fusion experiments. *In re Wands*, 858 F.2d 731, 783-39 (Fed. Cir. 1988). The single alleged inoperative embodiment relied on by the Examiner is a much lower percentage of the sequences tested by Fearon et al. than the 40% of failed experiments and 56% of non-binding antibodies upheld by the court as not precluding enablement of the claimed inventions in *Atlas Powder* and *In re Wands*, respectively.

b. Immunostimulatory activity was evaluated in a different context by Fearon et al. than the presently claimed methods.

The experiments performed by Fearon et al. were directed to optimization of immunostimulatory sequence requirements in an *in vitro* assay system. The system used by Fearon

et al. did not include an ISS in the same context as the methods and compositions of the instant claims. The instant claims are directed to methods and compositions comprising (i) a complex comprising an immunomodulatory polynucleotide (comprising an ISS) *covalently conjugated to a first antigen* and (ii) *a second antigen*. The assay system used by Fearon et al. contained neither a covalent complex of first antigen and ISS nor a second antigen. Fearon et al. used an ISS polynucleotide *adsorbed* to the surface of a *polymer*, poly(D,L-lactide-co-glycolide) microspheres (cPLGA). Fearon et al., page 2215, first complete paragraph in left-hand column, and page 2120, paragraph under subheading numbered 4.3. Fearon et al. did not use a covalent conjugate of ISS to polymer, much less a covalent complex with an antigen as claimed. Further, Fearon et al. evaluated effect of ISS sequence on cytokine production *in vitro*, rather than modulation of an immune response to a second antigen as claimed. Thus, the context of the experiments performed by Fearon et al. was different than the presently claimed invention.

In the 6/13/05 Office Action, the Examiner states "[t]he instant claims are drawn to a method that requires two components: (i) a complex comprising an immunomodulatory polynucleotide (ISS) that has been complexed with an antigen. Fearon et al. teach an ISS complexed to another molecule, cPLGA." 6/13/05 Office Action, pages 2-3. These statements by the Examiner fail to reflect the fact that the instant claims recite a **covalent** complex between an immunomodulatory polynucleotide and a first antigen. The Examiner further relates cPLGA to the claimed first antigen complexed with the immunomodulatory polynucleotide, but fails to provide any evidence that cPLGA is antigenic. Therefore, the ISS complexes taught by Fearon et al. differ from the claimed invention in two respects, (i) covalent conjugation, and (ii) conjugation to an antigen.

The Examiner goes on to state "[t]he instant claims additionally require a second component, (ii) a second antigen. Fearon et al. additionally teach the activity of ISS molecules that are uncomplexed. Therefore, free ISS and complexed ISS molecules of Fearon et al. are comparable to the instant ISS-complexes and antigens that are uncomplexed with ISS." 6/13/05 Office Action, page 3. Applicants do not understand the Examiner's reasoning. Fearon et al. do not include any antigen in their compositions, much less a conjugated first antigen and an unconjugated second antigen as instantly claimed. Further, the Examiner equates free ISS with the claimed

second antigen to which an immune response is modulated. However, even if, for the sake of argument only, free ISS were equivalent to the second antigen, Fearon do not disclose including free ISS (*i.e.*, the "second antigen") and complexed ISS in a single composition, with respect to the rejected composition claims, or co-administration of free ISS and complexed ISS in the *in vitro* assay system used by these authors, with respect to the rejected method claims. Thus, the Examiner's comments are not relevant to the instant claims.

3. An analysis of the factors set forth in In re Wands shows that the claimed invention is enabled.

With regard to the Examiner's assertion that the single inactive species in Fearon et al. "[exemplifies] the unpredictability of immunostimulatory sequences," Applicants note that unpredictability is only one of several factors which must be weighed in an enablement analysis. MPEP § 2164.01(a) lists the factors to be considered, as set forth in *In re Wands*, 858 F.2d 731,737 (Fed. Cir. 1988). These factors include: (A) the breadth of the claims; (B) the nature of the invention; (C) the state of the prior art; (D) the level of one of ordinary skill; (E) the level of predictability in the art; (F) the amount of direction provided by the inventor; (G) the existence of working examples; and (H) the quantity of experimentation needed to make or use the invention based on the content of the disclosure.

The MPEP states that "[i]t is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others." Here, the Examiner considered only one of eight factors and ignored the other seven. The factors must all be considered in an enablement analysis; no one factor is dispositive. In the 6/13/05 Office Action, the Examiner repeatedly refers to predictability (factor E) and mentions quantity of experimentation (factor H), but does not analyze the enablement of Applicants' invention in terms of the other factors, as required by the court's decision in *In re Wands* and the MPEP. A rejection based on one or two of these factors alone, while ignoring the other factors, is improper. The Examiner discussed enablement of the invention in view of the *Wands* factors in the Office Action dated 6/2/03, but this analysis related to a previous enablement rejection against the method claims which has since been withdrawn. The previous rejection, and hence the previous enablement analysis, have since been

rendered moot in view of subsequent amendments that were made to the claims. The Examiner has not reapplied this analysis in view of the current enablement rejection in which the Fearon et al. reference has been used to support the rejection.

Applicants therefore provide the following enablement analysis using the factors set forth in *In re Wands*:

A. With respect to the breadth of the claims, the claims are directed to novel methods and compositions that include immunostimulatory sequences. Immunostimulatory sequences are known in the art and may be identified and tested using techniques that are well-established in the art.

B. With regard to the nature of the invention, the invention relates to methods and compositions for modulating an immune response to a second antigen by co-administering a complex comprising an ISS polynucleotide covalently conjugated to a first antigen and a second antigen. Methods for assessing modulation of an immune response are well known in the art, and a working example is provided which exemplifies the claimed methods.

C and D. As discussed above, the state of the prior art (factor C) and the level of one of ordinary skill (factor D) are high, because much has been written in both the scientific and patent literature about how to make and use immunostimulatory sequences in several species e.

E. With regard the level of predictability in the art, it is predictable that many sequences within the parameters set forth in the specification are operable in the claimed invention, as demonstrated by the working example in the specification and the many active immunostimulatory sequences described by Fearon et al. (see, *e.g.*, Figs. 2. and 3). The Examiner has relied on a single disclosed inactive polynucleotide sequence in Fearon et al. as the basis of an assertion that the ISS art is unpredictable. Fearon et al. described experiments designed to optimize ISS activity. The cited reference by Fearon et al. is focused on optimization and refinement of immunostimulatory sequence requirements rather than identification of sequences with some, if not optimal activity. Applicants submit that optimizability is not the same as predictability. The fact that the activity of a polynucleotide may be fine-tuned by sequence adjustments does not indicate unpredictability or lack of enablement. Fearon et al. teach one of many sequences that lacks activity, in the context of

many active sequences and optimization of activity through experimentation. Thus, the disclosure of one inactive sequence, especially in a different context than the claimed methods and compositions, does not equate with unpredictability.

F and G. With respect to the amount of direction provided by the inventor (factor F) and the existence of working examples (factor G), Applicants disclosed a working example in the specification, showing that the methods of the invention work as claimed, and guidance is provided in the specification regarding how to identify and assess additional ISS polynucleotide for use in the claimed methods.

H. With respect to the quantity of experimentation needed to make or use the invention based on the content of the disclosure, a number of examples of ISS polynucleotides are provided in the specification, including one which is exemplified in the claimed method in a working example, as well as disclosure teaching how to identify and evaluate other immunostimulatory polynucleotide sequences. Further, numerous ISS are known in the art, as well as techniquest to test them for immunostimulatory activity, for which references are provided in the specification and incorporated by reference (page 6, lines 1-2).

In conclusion and in view of the foregoing, the amount of experimentation needed to needed to practice the claimed invention or make the claimed compositions is not undue.

4. Other issued U.S. patents contain claims that recite an ISS comprising the sequence 5'-cytosine, guanine-3'.

In response to the current enablement rejection based on scope of the claimed ISS, Applicants noted in their response to the 9/22/04 Final Office Action that "the Office has recently issued claims directed to methods of treating a mammal, a subject or an individual through administering an immunostimulatory or immunomodulatory polynucleotide comprising an ISS, wherein the ISS comprises the sequence 5'-C,G-3'." Response with Request for Continued Examination filed on 12/22/04, page 10. Claims from the issued patents referred to by Applicants are reproduced below:

Claim 1 of U.S. Patent No. 6,498,148 recites:

> 1. A method for treating asthma, comprising: administering to a mammal sensitized to an asthma-stimulating antigen an immunostimulatory polynucleotide comprising an immunostimulatory sequence (ISS), wherein *the ISS comprises the sequence* ***5'-cytosine-guanine-3'***, wherein the immunostimulatory polynucleotide does not comprises a nucleotide sequence encoding the antigen, and wherein the immunostimulatory polynucleotide is administered without the antigen, including without a polynucleotide encoding the antigen, and in an amount sufficient to treat asthma.

Claims 1 and 2 of U.S. Patent No. 6,534,062 recite:

> 1. A method of increasing antigen-specific T lymphocyte activity in a CD4+ T cell-deficient individual, comprising administering a formulation *comprising an immunostimulatory nucleic acid molecule* and an antigen in an amount effective to increase antigen-specific CTL activity, wherein the immunostimulatory nucleic acid is covalently linked to the antigen.
>
> 2. The method of claim 1, wherein *the immunostimulatory nucleic acid comprises the sequence* ***5' C-G 3'***.

Claim 1 of U.S. Patent No. 6,552,006 recites:

> 1. A method for treating mycobacterial infection in a subject, the method comprising: administering to a subject an immunomodulatory nucleic acid molecule in an amount effective to inhibit intracellular replication of the mycobacterium, wherein *the immunomodulatory nucleic acid comprises an immunostimulatory sequence comprising* ***5' CpG 3'***; and administering to the subject an anti-pathogenic agent in an amount effective to decrease or inhibit growth of the mycobacterium, thereby treating the infection.

Claim 1 of U.S. Patent No. 6,613,751 recites:

> 1. A method for ameliorating gastrointestinal inflammation in a subject comprising: administering to a subject suffering from gastrointestinal inflammation a formulation comprising an

immunomodulatory nucleic acid to the subject, *the immunomodulatory nucleic acid comprising the sequence* ***5'-CpG-3',*** wherein said immunomodulatory nucleic acid is isolated or synthetic, said administering being in an amount effective to ameliorate a symptom of gastrointestinal inflammation in the subject; wherein said administering is by a route selected from oral and subcutaneous, and wherein gastrointestinal inflammation is ameliorated in the subject.

The claims above recite an ISS comprising a 5'-CG-3' sequence, except claim 1 of 6,534,062, which is of even broader scope and recites "an immunostimulatory nucleic acid molecule," with no recited sequence requirements. In response to Applicants' arguments with respect to these issued patents, the Examiner stated that "[t]he patents discussed are not relevant to the instant case since the method of treatment is unrelated and the claimed compositions do not resemble the one instantly claimed." 6/13/05 Office Action, page 4. Applicants disagree that these patents are not relevant, since the current rejection is based on the scope of the claimed ISS and the alleged unpredictability of immunostimulatory activity, not the "method of treatment" or the "claimed compositions" *per se*. The Examiner discusses the alleged lack of enablement of the present invention in terms of the alleged lack of immunostimulatory activity of a single polynucleotide sequence in Fearon et al., not in terms of the actual recited steps of the claimed method or the components of the claimed compositions other than the ISS. The issued patents referenced above contain claims reciting ISS of the same or broader scope than the instant claims, and the USPTO found claims reciting ISS of such scope to be enabled and therefore not unpredictable to the extent that the respective inventions were not enabled, in contrast to the assertions by the Examiner in the instant case. Each of these patents contains claims reciting an ISS of identical scope and breadth as (or broader than) the ISS of the instant claims comprising the sequence 5'-C,G-3' and was found by the USPTO to be in compliance with 35 U.S.C. §112, first paragraph.

B. Claims 1, 13, 14, 17, 20-23, 25-33, 37, and 40-42 are patentable under 35 U.S.C. §103 over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997).

With respect to this rejection, claims 1, 13, 14, 17, 20-23, 25-33, 37, and 40-42 stand or fall separately as indicated below.

Applicants respectfully submit that for each of the rejected claims, the Examiner has not established a *prima facie* case for obviousness in view of the cited references.

CLAIMS 1, 13, 25-33 AND 42

1. The Examiner has failed to establish a prima facie case for obviousness.

A *prima facie* case of obviousness requires that three basic criteria must be met. First, the prior art reference (or references when combined) must teach or suggest all the claim limitations. Second, there must be some suggestion of motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify a reference or to combine reference teachings. Finally, there must be a reasonable expectation of success. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, not in the applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991); MPEP §2143.

All three elements of a *prima facie* case must be present in order for the Office to meet its burden. If any one of these three criteria is not satisfied, a *prima facie* case of obviousness has not been established. In this instant case, none of these three criteria is satisfied by the cited combination of references. That is, all limitations of the claimed invention are not found in the cited references, either singly or in combination; there is neither an explicit suggestion to modify the cited references nor a chain of reasoning that would have led one of skill in the art to make such modification in the absence of an explicit suggestion; and there would not have been a reasonable expectation of success at the application's filing date.

The present invention is based on the observed benefit of co-administration of an immunomodulatory polynucleotide-first antigen complex with a second antigen in the *modulation of an immune response to the second antigen*. As demonstrated in this application, administration of an ISS-first antigen complex with a second antigen results in an enhanced Th1 immune response to the second antigen as compared to a Th1 response that would be obtained upon administration of the second antigen with an ISS in an admixture and as compared to the immune response to the administration of the second antigen without an ISS. None of the cited references teaches modulation of an immune response to a second antigen by administering an ISS-containing polynucleotide conjugated to a first antigen, and none of the references teach co-administration of a second antigen in conjunction with an immunomodulatory polynucleotide-first antigen conjugate as claimed. Further, none of the references suggests or provides motivation for administering a second antigen and modifying the immune response to the second antigen, and there would have been no reasonable expectation of success for one of skill in the art to do so at the time of filing of this application.

a. The cited references do not reach or suggest each and every limitation of the claimed invention, either singly or in combination.

None of the cited references, or a combination thereof, teaches all of the elements of the claimed invention.

Claim 1, upon which the other claims in this group are dependent, recites a method of modulating an immune response to a second antigen through co-administration of (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and (ii) a second antigen, where the polynucleotide comprises an ISS comprising the sequence 5'-cytosine, guanine-3', where the complex and the second antigen are administered at the same site, and where the amount of the complex administered is sufficient to modulate an immune response to the second antigen.

Claim 13 recites that the first antigen (*i.e.*, the antigen that is covalently conjugated to the immunomodulatory polynucleotide) is an allergen. Claim 42 further recites that the allergen is Amb a I.

Claims 25-31 recite that the ISS comprises the sequence 5'-TCG'3', 5'-purine, purine, C, G, pyrimidine, pyrimidine-3', 5'-AACGTT-3', 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3', 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3', is selected from the group consisting of AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG, or comprises TGACTGTGAACGTTCGAGATGA (SEQ ID NO:1), respectively.

Claims 32 and 33 recite that the individual in whom the immune response to a second antigen is modulated is a mammal or human, respectively.

i. Deficiencies of Schwartz et al., PCT Application No. WO 98/55495 ("Schwartz")

Schwartz does not teach all of the elements of the claimed invention. Schwartz does not teach co-administration of (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and (ii) a second antigen, where the amount of the polynucleotide and first antigen administered is sufficient to modulate an immune response to the second antigen. Schwartz teaches an ISS-first antigen conjugate, but *does not teach administration of or modulation of an immune response to a second antigen.*

The Examiner states that "[t]he instant claims are drawn to a method with two components: (i) an ISS-antigen complex and (ii) [an] un-complexed second antigen. On page 12, lines 29-31, Schwartz states that '[t]he ISS and the antigen . . . can be administered together in the form of a conjugate' (this teaching embraces (i) of the instant claims) 'or co-administered in an admixture sufficiently close in time so as to modulate an immune response' (since the instant un-complexed second antigen is co-administered with the ISS complex, this phrase embraces (ii) of the instant claims). Schwartz also discussed these same concepts again on page 14, lines 8-14. Therefore, Schwartz et al. do suggest the instant composition claimed." 6/13/05 Office Action, page 6.

These statements by the Examiner are in direct contradiction to statements made in a previous Office Action, where the Examiner stated that "Schwartz does not explicitly teach administering a second antigen with the composition." 4/23/02 Office Action, page 3. Further, Applicants disagree with the Examiner's reasoning in the 6/13/05 Final Office Action, quoted

above. The Examiner points to pages from Schwartz that are alleged to disclose either component (i) ***or*** component (ii), and fails to point to any disclosure in Schwartz that teaches co-administration of components (i) and (ii) as claimed. Further, Applicants disagree with the Examiner's characterization of the disclosure of Schwartz. The passage quoted by the Examiner discloses administration of an ISS and an antigen in the form of a conjugate (component (i) of the instant claims) ***or*** in the form of an admixture (neither component (i) nor (ii); the instant claims require an immunomodulatory polynucleotide-antigen conjugate, and do not recite free, unconjugated ISS in admixture with an antigen)). The cited passage from Schwartz does not disclose administration of a second antigen (component (ii)) in conjunction with component (i) as claimed. The Examiner has failed to point to any disclosure in Schwartz regarding administration of both components (i) and (ii) as claimed.

ii. Deficiencies of Carson et al., PCT Application No. WO 98/16247 ("Carson")

As with Schwartz, discussed above, Carson does not teach all of the elements of the claimed invention. Carson does not teach co-administration of (i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and (ii) a second antigen, where the amount of the polynucleotide and first antigen administered is sufficient to modulate an immune response to the second antigen. Carson teaches an ISS polynucleotide-first antigen conjugate, but *does not teach administration of or modulation of an immune response to a second antigen.*

In the Office Action dated June 2, 2003, the only Office Action in which the Examiner discussed the disclosure of Carson in detail, the Examiner stated that "Carson teaches an immunomodulatory composition comprising an ISS conjugated to AgE, also known as amb a1, see page 19, line 22, and Figures 3-5. Carson also teaches ISS partners include antigens from viruses, see page 18, lines 10-12. . . . Although *Carson does not explicitly teach administering a second antigen with the composition*, Carson teaches that ISS molecules induce a Th-1 response to an antigen, see page 11, lines 21-28. Carson teaches administering an ISS-antigen complex and a mixture comprising ISS and an antigen to induce a Th-1 response to the antigen, see page 35, lines

10-17 and figure 1." 6/2/03 Office Action, paragraph bridging bottom of page 11 and top of page 12, emphasis added.

Nowhere in this description of Carson did the Examiner point to a single disclosure of administration of a second antigen in conjunction with an immunomodulatory polynucleotide-first antigen conjugate as claimed. Indeed, the Examiner admitted on the record that Carson does not teach administration of a second antigen. As with Schwartz, discussed above, Carson teaches an immunomodulatory polynucleotide-first antigen conjugate, but *does not teach administration of or modulation of an immune response to a second antigen.* The Examiner has not provided any citation from Carson to support Carson teaching or suggesting co-administration of an immunomodulatory-first antigen conjugate and a second antigen.

iii. The deficiencies of Schwartz et al. and Carson et al. are not cured by Horner et al. (*Cellular Immunology* 190:77-82, 1998) ("Horner") or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997) ("Chu").

Horner and Chu are cited as allegedly teaching a Th1 response is induced against an antigen co-administered with an ISS. See 3/9/04 Office Action, page 9. Neither of these references teaches or suggests modulating an immune response to a second antigen through co-administration of the second antigen and a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen. Thus, neither of these references cures the deficiencies of Schwartz or Carson, as discussed above, *i.e.*, lack of teaching or suggestion regarding administration of second antigen in conjunction with an immunomodulatory polynucleotide-first antigen conjugate.

Therefore, none of the cited references, either alone or in combination, describes or suggests the methods for modulating an immune response to a second antigen as claimed. None of the references, either alone or in combination, teaches all of the elements of the claimed invention, as required for establishment of a *prima facie* case for obviousness under 35 U.S.C. §103.

b. There is no suggestion or motivation to combine or modify the cited references.

A *prima facie* case for obviousness requires that there must be some suggestion of motivation, either in the references themselves or in the knowledge generally available to one of

ordinary skill in the art, to modify a cited reference or combine reference teachings. *In re Vaeck*, MPEP §2143.

The Examiner has failed to point out an explicit suggestion or motivation to combine the cited references to obtain every element of the claimed invention; indeed, this would be impossible since none of the references teach or suggest administration of a second antigen in conjunction with an immunomodulatory polynucleotide-first antigen conjugate, as discussed in detail above.

Further, there is no suggestion of motivation in the cited references to modify the teachings therein to arrive at the current invention. Nothing in the cited references provides motivation to the skilled artisan to administer an ISS-first antigen conjugate complex in an amount sufficient to modulate an immune response to a co-administered, but unconjugated, second antigen.

The present invention is based on the observed benefit of co-administration of an immunomodulatory polynucleotide-first antigen complex with a second antigen in the modulation of the immune response to the second antigen. Without an understanding of this benefit, one skilled in the art would have no motivation to undertake the claimed method. Both of the primary references, Schwartz and Carson, demonstrate that conjugation of an ISS molecule to an antigen is much more effective in stimulating an immune response to the antigen than administration of the antigen and ISS unconjugated in a mixture. Accordingly, based on the teachings of these references, *i.e.*, the knowledge in the art, the most effective method to stimulate an immune response to an antigen is to administer the antigen in the form of an ISS-antigen conjugate. Based on the teachings of these references, one of skill in the art would not be motivated to administer an antigen in a form in which it is not conjugated to an ISS. Nothing in Schwartz or Carson, or in the knowledge in the art at the time of filing, suggests that the benefit of conjugation of an antigen to an ISS would be extended to a second, co-administered but unconjugated, antigen.

In response to arguments by Applicants that the cited references do not provide motivation for one of skill in the art to modify the teachings therein to arrive at the claimed invention, the Examiner stated that "Applicant's arguments have been fully considered, but are found unpersuasive. The claims are drawn to modulating an immune response to a second antigen

by co-administering an ISS-antigen complex and a second antigen. The prior art cited clearly teaches that ISS-antigen complexes induce an immune response and that ISS/antigen mixtures induce an immune response. The immune response induced by an ISS/antigen mixture is an immuno-modulation against the unconjugated antigen, which is all that [is] required by the claims." 6/13/05 Office Action, page 6. As an initial matter, Applicants submit that an "immune response induced by an ISS/antigen mixture" is not equivalent to an immune response induced by an immunomodulatory polynucleotide-first antigen conjugate as claimed, and this is not "all that is required by the claims." The claims require co-administration of an immunomodulatory polynucleotide-antigen conjugate and a second antigen to modulate an immune response against the second antigen. The above statements by the Examiner fail to address the fundamental issue that none of the cited reference teaches co-administration of an ISS-antigen conjugate **and a second antigen**. The statements by the Examiner fail to point to a single disclosure within the cited references or a single piece of evidence of knowledge in the art at the time of filing that would lead a skilled artisan to modify the cited references to practice the method as claimed.

c. The cited references do not provide a reasonable expectation of success with respect to the claimed methods.

The third requirement for a *prima facie* showing of obviousness is that one of ordinary skill in the art must have had a reasonable expectation of success in practicing the claimed invention at the time of filing. *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991); MPEP §2143.02. The Applicant may adduce evidence that such an expectation of success was not present that, especially if unchallenged by the Examiner, tips the balance towards non-obviousness. *In re Rinehart*, 531 F.2d 1048, (CCPA 1976); *Amgen, Inc. V. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 1207-8, (Fed. Cir. 1991). In the present case, nothing in the cited references or the knowledge in the art at the time of filing would have provided a skilled artisan with a reasonable expectation of success in practicing the claimed invention, as required for a *prima facie* case for obviousness.

i. All limitations of the claims are not taught in the cited references.

It is a threshold requirement that all limitations be present in the cited references before it is even relevant whether there was a reasonable expectation of success by one of ordinary skill in

the art. In the present case, as discussed above, the cited references do not disclose all of the elements of the claimed invention, either singly or in combination. Therefore, there could have been no reasonable expectation of success, since one of skill in the art could not have discerned each and every limitation of the claimed invention in the cited references.

ii. The cited references did not predict the results of the claimed methods, let alone success of these methods, at the time of filing.

From the teachings in the cited primary references Schwartz and Carson, one of skill in the art would expect an enhanced immune response to an antigen when it is conjugated to an ISS. In view of this teaching, one of skill in the art would not predict that an immune response to a second, unconjugated antigen would be modulated. These references do not provide a reasonable expectation of success that administration of an ISS-containing polynucleotide covalently conjugated to a first antigen would modulate an immune response to an *unconjugated* second antigen, including stimulating a Th1 response to a second administered antigen.

On October 23, 2002, Applicants submitted a 37 C.F.R. §1.132 Declaration from co-inventor Dr. Gary Van Nest, which provided additional support that the immune response to an antigen following the claimed method (administration of (i) an ISS-containing polynucleotide conjugated to a first antigen and (ii) a second antigen) is different from the immune response to the antigen (*i.e.*, second antigen) following the control method (administration of (i) an ISS-containing polynucleotide and (ii) the antigen (*i.e.*, second antigen)).

Dr. Van Nest's Declaration provides results of controls performed along with the experiments presented in Example 1 of the specification. In these controls, the antigen (βgal) was administered in an admixture with the ISS-containing polynucleotide and the immune response to the antigen was measured. Comparison of the βgal-specific immune response obtained using the claimed method with the βgal-specific immune response obtained with the controls clearly indicates that the claimed method results in a modulation of an immune response to the βgal antigen compared to that of the control. A copy of the Declaration of Dr. Van Nest is provided herewith in Appendix B. The Declaration of Dr. Van Nest shows a result that was unexpected and not predictable by the cited references and the knowledge in the art at the time of filing. Accordingly,

the cited references provide no reasonable expectation of success of the claimed invention and thus do not support *prima facie* obviousness with regard to the claimed invention.

CLAIM 14

Claim 14 is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 14 be rendered obvious thereby.

Further, claim 14 recites that "the first antigen is a conserved polypeptide of a virus." The Examiner failed to discuss this limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. In the 6/2/03 Office Action, on page 9, the Examiner stated that "Schwarz also teaches antigenic peptides derived from viruses are also administered in conjunction with an ISS sequence, see page 13, line 26 to page 14, line 3." However, the Examiner did not address the specific limitation of claim 14, *i.e.*, a "*conserved* polypeptide of a virus." Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 17

Claim 17 is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 17 be rendered obvious thereby.

Further, claim 17 recites that "the first antigen is a carrier molecule." The Examiner failed to discuss this limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 20

Claim 20 is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 20 be rendered obvious thereby.

Further, claim 20 recites that "the first antigen is associated with a carrier molecule." The Examiner failed to discuss this limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 21

Claim 21 is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 21 be rendered obvious thereby.

Further, claim 21 recites that "the immune response [to the second antigen] is modulated by stimulating a Th1 response to the second antigen." As discussed above, the cited references do not teach administration and modulation of an immune response to a second antigen co-administered with an immunomodulatory polynucleotide-antigen conjugate. Since the references do not teach administration or modulation of an immune response to a second antigen, it follows that they do not teach stimulation of any immune response to the second antigen, including a Th1 immune response as claimed. The Examiner states that "Carson, Horner et al. or Chu et al. specifically teach inducing a specific Th1 response to an antigen that is co-administered with an ISS molecule." 3/9/04 Office Action, page 9. However, the claims recite co-administration of a complex comprising a immunomodulatory polynucleotide (comprising an ISS) covalently conjugated to a first antigen in conjunction with a second antigen, **not** "an antigen that is co-administered with an ISS molecule" as described by the Examiner. Therefore, the Examiner has not discussed the limitation of claim 21 in terms that relate to the invention as claimed, and thus has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 22

Claim 22 is dependent on claim 21, which is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 22 be rendered obvious thereby.

Further, claim 22 recites that "production of second antigen-specific Th1-associated antibodies is stimulated." The Examiner failed to discuss this limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. Also, as discussed above with respect to claim 21, the Examiner has failed to show that any of the cited references teach administration of a second antigen in conjunction with an ISS-first antigen conjugate, and therefore has not shown that the art teaches that any immune response is generated to such a second antigen, much less a Th1 response, such as stimulation of production of second antigen-specific Th1-associated antibodies as claimed. Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 23

Claim 23 is dependent on claim 21, which is dependent on claim 1. As shown above, claim 1 is not rendered obvious by the cited references. Thus, neither can claim 23 be rendered obvious thereby.

Further, claim 21 recites that "interferon gamma production is stimulated." The Examiner failed to discuss this limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. Also, as discussed above with respect to claim 21, the Examiner has failed to show that any of the cited references teach administration of a second antigen in conjunction with an ISS-first antigen conjugate, and therefore has not shown that the art teaches that any immune response is generated to such a second antigen, much less a Th1 response, such as stimulation of interferon gamma production as claimed. Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIM 37

Claim 37 recites a composition comprising a complex comprising (i) an immunomodulatory polynucleotide covalently conjugated to a first antigen and (ii) a second

antigen, wherein the polynucleotide comprises an ISS comprising the sequence 5'-cytosine, guanine-3', and wherein the first antigen is a viral conserved polypeptide and the second antigen is a viral variable polypeptide.

As discussed in detail above, none of the cited references teaches or suggests a second antigen in combination with an immunomodulatory polynucleotide-first antigen conjugate as claimed, none of the cited reference provides motivation for modification of the teachings therein to include a second antigen in combination with an ISS-antigen conjugate, and there would have been no reasonable expectation of success for one of skill in the art to use such a combination to modulate an immune response to the second antigen. Therefore, the Examiner has failed to establish a *prima facie* case for obviousness. In addition, the Examiner failed to discuss the claim limitations that the first antigen is a viral conserved polypeptide and the second antigen is a viral variable polypeptide in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. In the 6/2/03 Office Action, on page 9, the Examiner stated that "Schwarz also teaches antigenic peptides derived from viruses are also administered in conjunction with an ISS sequence, see page 13, line 26 to page 14, line 3." However, the Examiner did not address the specific limitation of claim 37, *i.e.*, a "viral *conserved* polypeptide" or a "viral *variable* polypeptide," with respect to the first antigen and second antigen, respectively. Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

CLAIMS 40 AND 41

Claim 40 recites a composition comprising a complex comprising (i) an immunomodulatory polynucleotide covalently conjugated to a first antigen and (ii) a second antigen, wherein the polynucleotide comprises an ISS comprising the sequence 5'-cytosine, guanine-3', and wherein the first antigen is an allergen.

Claim 41 recites that the allergen is Amb a I.

As discussed in detail above, none of the cited references teach or suggest a second antigen in combination with an immunomodulatory polynucleotide-first antigen conjugate as claimed, none of the cited reference provide motivation for modification of the teachings therein to include a second antigen in combination with an ISS-antigen conjugate, and there would have been no reasonable expectation of success for one of skill in the art to use such a combination to modulate an immune response to the second antigen. Therefore, the Examiner has failed to establish a *prima facie* case for obviousness.

C. Claims 15 and 38 are patentable under 35 U.S.C. §103 over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Lee et al. (*Ann. Med.* 30:460-468, 1998).

With respect to this rejection, claims 15 and 38 stand or fall separately as indicated below.

CLAIM 15

Claim 15 is dependent on claim 14, which is dependent on claim 1. As discussed in detail above, claim 1 is not rendered obvious by the cited references Schwartz, Carson, Horner, and Chu. Claim 14 recites the limitation that "the first antigen is a conserved polypeptide of a virus," and claim 15 further recites that the conserved viral polypeptide is influenza nucleocapsid protein.

The Examiner states that the primary references, Schwartz, Carson, Horner, and Chu, "each . . . individually teaches inducing a Th-1 response against an antigen present in a mixture with an ISS molecule or in an ISS-antigen complex. Therefore, Lee is only required to teach a limitation that is not taught by the primary references." 9/22/04 Office Action, page 12. Applicants disagree with the Examiner's reasoning. As discussed in detail above, none of the primary references teaches modulation of an immune response to a second antigen that is co-administered with an ISS-first antigen conjugate, nor provides a motivation to modify the teachings therein to arrive at the

claimed invention, nor a reasonable expectation of success in practicing the claimed invention. Lee et al. (1998) *Ann. Med.* 30:460-468 ("Lee") do not cure these deficiencies of Schwartz, Carson, Horner, and Chu.

The Examiner states that "Lee et al. teach that the influenza nucleocapsid protein is the least effected by antibody-induced antigenic drift and studies using DNA encoding this protein have demonstrated protection, see "infectious diseases" on page 465. One of ordinary skill in the art would have been motivated to incorporate a protein into a treatment composition that has already demonstrated protective properties in other studies. Furthermore, one of ordinary skill in the art would have had a reasonable expectation in producing the claimed invention because Schwartz or Carson teach compositions and methods comprising ISS and proteins that modulate the immune response and Lee et al. also teach subsequent Th1 responses upon administration of ISS with DNA encoded antigens, se "mechanism of action . . ." on pages 463-464. Therefore, the invention as a whole is prima facie obvious to one of ordinary skill in the art at the time the invention was made, absent unexpected results." 9/22/04 Office Action, page 12.

The Examiner's reasoning is faulty because nowhere does she address the fundamental deficiency in all of the cited references, *i.e.*, *the lack of any teaching regarding administration of and modulation of an immune response to a second antigen by co-administering an immunomodulatory polynucleotide-first antigen conjugate*. The Examiner discusses an alleged disclosure by Schwartz or Carson of compositions and methods comprising ISS and proteins that modulate the immune response, but the claim does not recite free ISS alone co-administered with an antigen. The Examiner discusses an alleged disclosure by Lee of a Th1 response to administration of an ISS with a DNA encoded antigen, but the claim does not recite free ISS co-administered with antigen. Rather, claim 1, from which claim 15 depends, recites co-administration of a second antigen with an immunomodulatory polynucleotide-first antigen conjugate.

Therefore, the Examiner has failed to establish a *prima facie* case for obviousness under 35 U.S.C. §103 with respect to claim 15.

CLAIM 38

Claim 38 is dependent on claim 37. As discussed in detail above, claim 37 is not rendered obvious by the cited references Schwartz, Carson, Horner, and Chu. Claim 38 recites the limitation that "the first antigen is influenza nucleocapsid protein.

The Examiner states that the primary references, Schwartz, Carson, Horner, and Chu, "each . . . individually teaches inducing a Th-1 response against an antigen present in a mixture with an ISS molecule or in an ISS-antigen complex. Therefore, Lee is only required to teach a limitation that is not taught by the primary references." 9/22/04 Office Action, page 12. Applicants disagree with the Examiner's reasoning. As discussed in detail above, none of the primary references teaches a second antigen that in combination with an ISS-first antigen conjugate, nor provides a motivation to modify the teachings therein to arrive at the claimed composition, nor a reasonable expectation of success in using the claimed composition in the claimed methods. Lee et al. (1998) *Ann. Med.* 30:460-468 ("Lee") do not cure these deficiencies of Schwartz, Carson, Horner, and Chu.

The Examiner states that "Lee et al. teach that the influenza nucleocapsid protein is the least effected by antibody-induced antigenic drift and studies using DNA encoding this protein have demonstrated protection, see "infectious diseases" on page 465. One of ordinary skill in the art would have been motivated to incorporate a protein into a treatment composition that has already demonstrated protective properties in other studies. Furthermore, one of ordinary skill in the art would have had a reasonable expectation in producing the claimed invention because Schwartz or Carson teach compositions and methods comprising ISS and proteins that modulate the immune response and Lee et al. also teach subsequent Th1 responses upon administration of ISS with DNA encoded antigens, see "mechanism of action . . ." on pages 463-464. Therefore, the invention as a whole is prima facie obvious to one of ordinary skill in the art at the time the invention was made, absent unexpected results." 9/22/04 Office Action, page 12.

The Examiner's reasoning is faulty because nowhere does she address the fundamental deficiency in all of the cited references, *i.e., the lack of any teaching regarding a second antigen in combination with an immunomodulatory polynucleotide-first antigen conjugate.* The Examiner discusses an alleged disclosure by Schwartz or Carson of compositions and methods comprising ISS

and proteins that modulate the immune response, but the claim does not recite free ISS in combination with an antigen. The Examiner discusses an alleged disclosure by Lee of a Th1 response to administration of an ISS with a DNA encoded antigen, but the claim does not recite free ISS in combination with antigen. Rather, claim 37, from which claim 38 depends, recites a second antigen in combination with an immunomodulatory polynucleotide-first antigen conjugate.

Further, claim 37 recites that "the second antigen is a viral variable polypeptide." The Examiner failed to discuss this claim limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. In the 6/2/03 Office Action, on page 9, the Examiner stated that "Schwarz also teaches antigenic peptides derived from viruses are also administered in conjunction with an ISS sequence, see page 13, line 26 to page 14, line 3." However, the Examiner did not address the specific limitation of claim 37, *i.e.*,. a "viral *variable* polypeptide." Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

In conclusion, the Examiner has failed to establish a *prima facie* case for obviousness under 35 U.S.C. §103 with respect to claim 38.

D. Claims 16 and 39 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Durali et al. (*Journal of Virology* 72(5): 3547-3553, 1998).

With respect to this rejection, claims 16 and 39 stand or fall separately, as indicated below.

CLAIM 16

Claim 16 is dependent on claim 14, which is dependent on claim 1. As discussed in detail above, claim 1 is not rendered obvious by the cited references Schwartz, Carson, Horner, and Chu. Claim 14 recites the limitation that "the first antigen is a conserved polypeptide of a virus,"

and claim 16 further recites that "the conserved viral polypeptide is human immunodeficiency virus (HIV) gag protein."

The Examiner states that the primary references, Schwartz, Carson, Horner, and Chu, "each . . . individually teaches inducing a Th-1 response against an antigen present in a mixture with an ISS molecule or in an ISS-antigen complex. Therefore, Durali et al. is only required to teach a limitation that is not taught by the primary references." 9/22/04 Office Action, page 13. Applicants disagree with the Examiner's reasoning. As discussed in detail above, none of the primary references teaches modulation of an immune response to a second antigen that is co-administered with an immunodulatory polynucleotide-first antigen conjugate, nor provides a motivation to modify the teachings therein to arrive at the claimed invention, nor a reasonable expectation of success in practicing the claimed invention. Durali et al. (1998) *Journal of Virology* 72(5): 3547-3553 ("Durali") do not cure these deficiencies of Schwartz, Carson, Horner, and Chu.

The Examiner states that "Durali et al. teach that the gag protein is capable of cross-reactivity in different patients infected with different clades of HIV, see the abstract. Since high variability in HIV is a major obstacle in selecting an antigen for a vaccine candidate and Durali et al. have been able to identify a conserved protein, one of ordinary skill in the art would be motivated to incorporate this protein into a composition to induce an immune response against the antigen. Furthermore, the skilled artisan would have a reasonable expectation in producing the claimed invention because Schwartz et al. or Carson et al. teach that the protein portion of the composition and method could be a wide variety of proteins from viruses." 9/22/04 Office Action, page 13.

The Examiner's reasoning is faulty because nowhere does she address the fundamental deficiency in all of the cited references, *i.e.*, *the lack of any teaching regarding administration of and modulation of an immune response to a second antigen by co-administering an immunomodulatory polynucleotide-first antigen conjugate*. None of the cited references, either singly or in combination, teaches co-administration of a second antigen with an immunomodulatory polynucleotide-first antigen conjugate, as recited in claim 1, from which claim 16 depends. Further, Durali does not provide a motivation to modify the teachings of the primary references to arrive at

the claimed invention or a reasonable expectation of success in practicing the claimed method. Therefore, the Examiner has failed to establish a *prima facie* case for obviousness under 35 U.S.C. §103 with respect to claim 16.

CLAIM 39

Claim 39 is dependent on claim 37. As discussed in detail above, claim 37 is not rendered obvious by the cited references Schwartz, Carson, Horner, and Chu. Claim 39 recites the limitation that "the first antigen is human immunodeficiency virus (HIV) gag protein."

The Examiner states that the primary references, Schwartz, Carson, Horner, and Chu, "each . . . individually teaches inducing a Th-1 response against an antigen present in a mixture with an ISS molecule or in an ISS-antigen complex. Therefore, Durali et al. is only required to teach a limitation that is not taught by the primary references." 9/22/04 Office Action, page 13. Applicants disagree with the Examiner's reasoning. As discussed in detail above, none of the primary references teaches modulation of an immune response to a second antigen that is co-administered with an ISS-first antigen conjugate, nor provides a motivation to modify the teachings therein to arrive at the claimed invention, nor a reasonable expectation of success in practicing the claimed invention. Durali et al. (1998) *Journal of Virology* 72(5): 3547-3553 ("Durali") do not cure these deficiencies of Schwartz, Carson, Horner, and Chu.

The Examiner states that "Durali et al. teach that the gag protein is capable of cross-reactivity in different patients infected with different clades of HIV, see the abstract. Since high variability in HIV is a major obstacle in selecting an antigen for a vaccine candidate and Durali et al. have been able to identify a conserved protein, one of ordinary skill in the art would be motivated to incorporate this protein into a composition to induce an immune response against the antigen. Furthermore, the skilled artisan would have a reasonable expectation in producing the claimed invention because Schwartz et al. or Carson et al. teach that the protein portion of the composition and method could be a wide variety of proteins from viruses." 9/22/04 Office Action, page 13.

The Examiner's reasoning is faulty because nowhere does she address the fundamental deficiency in all of the cited references, *i.e.*, *the lack of any teaching regarding a second antigen in combination with an immunomodulatory-first antigen conjugate*. None of the cited references, either singly or in combination, teaches a second antigen in combination with an immunomodulatory polynucleotide-first antigen conjugate, as recited in claim 37, from which claim 39 depends. Further, Durali does not provide a motivation to modify the teachings of the primary references to arrive at the claimed invention or a reasonable expectation of success in practicing a method for modulating an immune response to a second antigen using the claimed composition.

Further, claim 37 recites that "the second antigen is a viral variable polypeptide." The Examiner failed to discuss this claim limitation in the 35 U.S.C. §103 rejection in the 7/30/01, 4/23/02, 6/2/03, 3/9/04, 9/22/04, or 6/13/05 Office Actions, or the 11/12/02 or 1/26/06 Advisory Actions. In the 6/2/03 Office Action, on page 9, the Examiner stated that "Schwarz also teaches antigenic peptides derived from viruses are also administered in conjunction with an ISS sequence, see page 13, line 26 to page 14, line 3." However, the Examiner did not address the specific limitation of claim 37, *i.e.*,. a "viral *variable* polypeptide." Therefore, she has not met her burden in establishing that this element is present in any of the cited references.

In conclusion, the Examiner has failed to establish a *prima facie* case for obviousness under 35 U.S.C. §103 with respect to claim 39.

E. Claims 18 and 19 are patentable under 35 U.S.C. §103(a) over Schwartz et al. (PCT Application No. WO 98/55495) or Carson et al. (PCT Application No. WO 98/16247), in view of Horner et al. (*Cellular Immunology* 190:77-82, 1998) or Chu et al. (*Journal of Experimental Medicine* 186(10): 1623-1631, 1997), and further in view of Anderson (U.S. Patent No. 4,673,574).

With respect to this rejection, claims 18 and 19 stand or fall together.

Claims 18 and 19 are dependent on claim 17, which is dependent on claim 1. As discussed in detail above, claim 1 is not rendered obvious by the cited references Schwartz, Carson,

Horner, and Chu. Claim 17 recites the limitation that "the first antigen is a carrier molecule" and claims 18 and 19 further recite that "the carrier molecule is diphtheria toxin mutant (CRM 197)" and that "the carrier molecule is diphtheria toxoid," respectively.

The Examiner states that the primary references, Schwartz, Carson, Horner, and Chu, "each . . . individually teaches inducing a Th-1 response against an antigen present in a mixture with an ISS molecule or in an ISS-antigen complex. Therefore, . . . Anderson is only required to teach a limitation that is not taught by the primary references." 9/22/04 Office Action, page 14. Applicants disagree with the Examiner's reasoning. As discussed in detail above, none of the primary references teaches modulation of an immune response to a second antigen that is co-administered with an ISS-first antigen conjugate, nor provides a motivation to modify the teachings therein to arrive at the claimed invention, nor a reasonable expectation of success in practicing the claimed invention. Anderson (U.S. Patent No. 4,673,574) does not cure these deficiencies of Schwartz, Carson, Horner, and Chu.

The Examiner states that "one of ordinary skill in the art at the time the invention was made would have been motivated to use the diphtheria components taught by Anderson in the method and composition taught by . . . Schwartz et al. or Carson et al. when administering the composition to children or immunocompromised individuals because the diphtheria toxins aid in eliciting a protective immune response, have no toxicity, and can be administered safely to children, see column 5, lines 10-19 and column 14, table 7. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation in producing the claimed invention because Schwartz et al. or Carson et al. teach that the ISS/antigen composition can be combined with any known vaccine component and the diphtheria toxins taught by Anderson are well known." 9/22/04 Office Action, page 14.

The Examiner's reasoning is faulty because nowhere does she address the fundamental deficiency in all of the cited references, *i.e.*, *the lack of any teaching regarding administration of and modulation of an immune response to a second antigen by co-administering an immunomodulatory polynucleotide-first antigen conjugate*. None of the cited references, either singly or in combination, teaches co-administration of a second antigen with an immunomodulatory

polynucleotide-first antigen conjugate, as recited in claim 1, from which claims 18 and 19 depend. Further, Anderson does not provide a motivation to modify the teachings of the primary references to arrive at the claimed invention or a reasonable expectation of success in practicing the claimed method. The Examiner discusses an alleged disclosure by Schwartz et al. and Carson et al. that an ISS/antigen composition can allegedly be combined with any known vaccine component, and alleges that this would have provided a reasonable expectation of success. However, the claims do not recite an "ISS/antigen composition." Rather, claim 1, from which claims 18 and 19 depend, recites co-administration of a second antigen with an immunomodulatory polynucleotide-first antigen conjugate.

Therefore, the Examiner has failed to establish a *prima facie* case for obviousness under 35 U.S.C. §103 with respect to claims 18 and 19.

VIII. CLAIMS APPENDIX

A copy of the claims involved in the present appeal is attached hereto as Appendix A.

IX. EVIDENCE APPENDIX

A declaration by Dr. Gary Van Nest pursuant to 37 C.F.R. § 1.132, and evidence entered by the Examiner and relied upon by Applicants in the appeal, are attached hereto as Appendix B.

X. RELATED PROCEEDINGS APPENDIX

No related proceedings are referenced in II, above. Hence, no Appendix is included.

Dated: July 12, 2006

Respectfully submitted,

By Jill A. Jacobson

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 09/642,492

Claim 1 (Previously presented): A method of modulating an immune response to a second antigen in an individual, comprising co-administering to the individual

(i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and

(ii) a second antigen

wherein the polynucleotide comprises an immunostimulatory sequence (ISS), wherein the ISS comprises the sequence 5'-cytosine, guanine-3', wherein the complex and the second antigen are administered at the same site in the individual and wherein the complex is administered in an amount sufficient to modulate an immune response in the individual to the second antigen.

Claims 2-12 (Canceled)

Claim 13 (Original): The method of claim 1, wherein the first antigen is an allergen.

Claim 14 (Original): The method of claim 1, wherein the first antigen is a conserved polypeptide of a virus.

Claim 15 (Original): The method of claim 14, wherein the conserved viral polypeptide is influenza nucleocapsid protein.

Claim 16 (Original): The method of claim 14, wherein the conserved viral polypeptide is human immunodeficiency virus (HIV) gag protein.

Claim 17 (Original): The method of claim 1, wherein the first antigen is a carrier molecule.

Claim 18 (Original): The method of claim 17, wherein the carrier molecule is diphtheria toxin mutant (CRM 197).

Claim 19 (Original): The method of claim 17, wherein the carrier molecule is diphtheria toxoid.

Claim 20 (Original): The method of claim 1, wherein the first antigen is associated with a carrier molecule.

Claim 21 (Original): The method of claim 1, wherein the immune response is modulated by stimulating a Th1 response to the second antigen.

Claim 22 (Previously presented): The method of claim 21, wherein production of second antigen-specific Th1-associated antibodies is stimulated.

Claim 23 (Original): The method of 21, wherein interferon gamma production is stimulated.

Claim 24 (Canceled)

Claim 25 (Previously presented): The method of claim 1, wherein the ISS comprises the sequence 5'-TCG-3'.

Claim 26 (Previously presented): The method of claim 1, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine-3'.

Claim 27 (Original): The method of claim 26, wherein the ISS comprises the sequence 5'-AACGTT-3'.

Claim 28 (Original): The method of claim 26, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, C-3'.

Claim 29 (Original): The method of claim 26, wherein the ISS comprises the sequence 5'-purine, purine, C, G, pyrimidine, pyrimidine, C, G-3'.

Claim 30 (Original): The method of claim 26, wherein the ISS comprises a sequence selected from the group consisting of AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG.

Claim 31 (Original): The method of claim 29, wherein the ISS comprises the sequence TGACTGTGAACGTTCGAGATGA (SEQ ID NO:1).

Claim 32 (Original): The method of claim 1, wherein the individual is a mammal.

Claim 33 (Original): The method of claim 32, wherein the mammal is human.

Claims 34-36 (Canceled)

Claim 37 (Previously presented): A composition comprising

(i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and

(ii) a second antigen,

wherein the polynucleotide comprises an immunostimulatory sequence (ISS), wherein the ISS comprises the sequence 5'-cytosine, guanine-3', and wherein the first antigen is a viral conserved polypeptide and the second antigen is a viral variable polypeptide.

Claim 38 (Previously presented): The composition of claim 37, wherein the first antigen is influenza nucleocapsid protein.

Claim 39 (Previously presented): The composition of claim 37, wherein the first antigen is human immunodeficiency virus (HIV) gag protein.

Claim 40 (Previously presented): A composition comprising

(i) a complex comprising an immunomodulatory polynucleotide covalently conjugated to a first antigen and

(ii) a second antigen,

wherein the polynucleotide comprises an immunostimulatory sequence (ISS), wherein the ISS comprises the sequence 5'-cytosine, guanine-3', and wherein the first antigen is an allergen.

Claim 41 (Previously presented): The composition of claim 40, wherein the allergen is Amb a I.

Claim 42 (Previously presented): The method of claim 13, wherein the allergen is Amb a I.

Claim 43 (Withdrawn): A method of treating an allergy in an individual, comprising administering to the individual an immunomodulatory polynucleotide comprising an immunostimulatory sequence (ISS) and a first allergen with a second allergen, wherein the ISS comprises the sequence 5'-cytosine, guanine-3', wherein the polynucleotide and the first allergen are proximately associated and wherein the polynucleotide and first allergen are administered in an amount sufficient to stimulate a Th1 immune response in the individual to the second allergen.

Claim 44 (Withdrawn): The method of claim 43, wherein the first allergen is Amb a I.

Claim 45 (Withdrawn): A method of vaccinating an individual, comprising administering to the individual an immunomodulatory polynucleotide comprising an immunostimulatory sequence (ISS) and a first antigen with a second antigen, wherein the ISS comprises the sequence 5'-cytosine, guanine-3', wherein the polynucleotide and the first antigen are proximately associated and wherein the polynucleotide and first antigen are administered in an amount sufficient to stimulate an immune response in the individual to the second antigen.

Claim 46 (Withdrawn): The method of claim 45, wherein the first antigen is a conserved polypeptide of a virus.

Claim 47 (Withdrawn): The method of claim 46, wherein the conserved viral polypeptide is influenza nucleocapsid protein.

Claim 48 (Withdrawn): The method of claim 46, wherein the conserved viral polypeptide is human immunodeficiency virus (HIV) gag protein.

Claim 49 (Withdrawn): The method of claim 45, wherein the first antigen is a carrier molecule.

Claim 50 (Withdrawn): The method of claim 49, wherein the carrier molecule is diphtheria toxin mutant (CRM 197).

Claim 51 (Withdrawn): The method of claim 49, wherein the carrier molecule is diphtheria toxoid.

Claim 52 (Withdrawn): The method of claim 45, wherein the first antigen is associated with a carrier molecule.

Claim 53 (Canceled)

APPENDIX B

Evidence Appendix

Tab	Reference
1.	Declaration of Gary Van Nest, Ph.D. Pursuant to 37 C.F.R. §1.132
2.	Fearon et al. (2003) *J. Immunol.* 33:2114-2122. "A minimal human immunostimulatory CpG motif that potently induces IFN-γ and IFN-α production"
3.	Schwartz et al., PCT Application No. WO 98/55495
4.	Carson et al., PCT Application No. WO 98/16247
5.	Horner et al. (1998) *Cellular Immunology* 190:77-82. "Immunostimulatory DNA is a Potent Mucosal Adjuvant"
6.	Chu et al. (1997) *Journal of Experimental Medicine* 168(10):1623-1631. "CpG Oligodeoxynucleotides Act as Adjuvants that Switch on T Helper 1 (Th1) Immunity"
7.	Lee et al. (1998) *Ann. Med.* 30:460-468. "Control of immune responses by gene immunization"
8.	Durali et al. (1998) *Journal of Virology* 72(5):3547-3553. "Cross-Reactions between the Cytotoxic T-Lymphocyte Responses of Human Immunodeficiency Virus-Infected African and European Patients"
9.	Anderson, U.S. Patent No. 4,673,574

PATENT
Docket No. 377882000800

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of: Gary VAN NEST, et al. Serial No.: 09/642,492 Filing Date: August 18, 2000 For: METHODS OF MODULATING AN IMMUNE RESPONSE USING IMMUNOSTIMULATORY SEQUENCES AND COMPOSITIONS FOR USE THEREIN	Examiner: S. Foley Group Art Unit: 1648

DECLARATION OF GARY VAN NEST, PH.D.
PURSUANT TO 37 C.F.R. § 1.132

Box AF
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Gary Van Nest, Ph.D., declare as follows:

1. I currently reside at 639 Skyline Drive, Martinez, California 94553.

2. I am an inventor named in the above-referenced patent application, and am familiar with the written communication from the Patent Office dated April 23, 2002.

3. Described herein are additional results from experiments, performed by me or under my direction, which are from controls performed along with the experiments described in Example 1 in the patent specification. These results support the difference in the immune

response to an antigen (*i.e.*, second antigen) following the claimed method (administration of (i) an ISS-containing polynucleotide in proximate association with a first antigen and (ii) a second antigen) from the immune response to the antigen (*i.e.*, second antigen) following the control method (administration of (i) an ISS-containing polynucleotide and (ii) the antigen (*i.e.*, second antigen)).

4. As described in Example 1 on page 50 of the specification, sets of mice were immunized with either 1 μg βgal, 1 μg βgal mixed with 1 μg AIC or 1 μg βgal mixed with 10 μg AIC. AIC denotes a conjugate of the antigen Amb a 1 and ISS-containing polynucleotide of SEQ ID NO:1. In addition to what is described in Example 1 of the specification, control sets of mice were immunized intradermally three times at two-week intervals with either 1 μg βgal mixed with 1 μg ISS or 1 μg βgal mixed with 10 μg ISS (admixture controls). Two weeks after the second and third immunizations, βgal-specific IgG1 and IgG2a responses were determined by ELISA as described in Example 1. Four weeks after the third immunization, mice were sacrificed, and spleen cell IFNγ and IL-5 responses to βgal were determined by ELISA as described in Example 1.

5. The results of this experiment with 1 μg βgal, 1 μg βgal mixed with 1 μg AIC, and 1 μg βgal mixed with 10 μg AIC are presented in Figure 1 and Tables 2 and 3 of the specification. The results of the admixture controls of this experiment with 1 μg βgal mixed with 1 μg ISS and with 1 μg βgal mixed with 10 μg ISS are herein presented in Exhibits A and B. The experimental data presented in the specification was calculated as arithmetic means as compared to the experimental data presented in Exhibits A and B which was calculated as geometric means.

6. After both the second and third immunizations, the antibody response to immunization with βgal alone was predominantly an IgG1 response, consistent with a Th2 response. Administration of βgal in an admixture with 1 or 10 μg of ISS-containing polynucleotide (admixture controls) resulted in a shift in the immune response away from a Th2 response and toward a Th1 response (Exhibit A). Co-administration of one or ten μg AIC (the ISS-containing polynucleotide linked to the antigen Amb a 1) with βgal modulated the βgal-specific immune response compared to the administration of βgal mixed with an ISS-containing polynucleotide (Exhibit A) and modulated the βgal-specific immune response compared to the

administration of βgal alone (specification, Figure 1 and Table 2). For example, modulation of a Th1 immune response was demonstrated by the increased IgG2a response to βgal after the second immunization with AIC as compared to the IgG2a response seen with βgal alone and as compared to the IgG2a response seen with βgal mixed with an ISS (admixture control) as depicted in Exhibit A.

7. As depicted in Table 3 of the specification and in Exhibit B, spleen cells from mice immunized with βgal alone secreted a relatively low level of IFNγ and a relatively high level of IL-5 in response to βgal. These cytokine responses are indicative of a Th2 response, consistent with the antibody responses discussed above. Administration of βgal in an admixture with 1 or 10 μg of ISS-containing polynucleotide resulted in a shift in the immune response away from a Th2 response and toward a Th1 response as indicated by an increased IFNγ and a decreased IL-5 in response to βgal (Exhibit B). Co-administration of βgal with 1 or 10 μg of AIC increased the IFNγ response and decreased the IL-5 response in response to βgal by the spleen cells (specification Table 3), again demonstrating a modulation of an immune response to βgal when the βgal is administered with AIC as compared to when the βgal is administered with an ISS-containing polynucleotide.

8. The data from this experiment, presented herein and in Example 1, demonstrate the difference in the immune response to an antigen (βgal) when administered with an ISS-containing polynucleotide (ISS) and when administered with an ISS-containing polynucleotide in proximate association with a different antigen (AIC). These admixture control results support the discovery that administration of a second antigen with an ISS-containing polynucleotide in proximate association to a first antigen results in a modulation of an immune response to the second antigen.

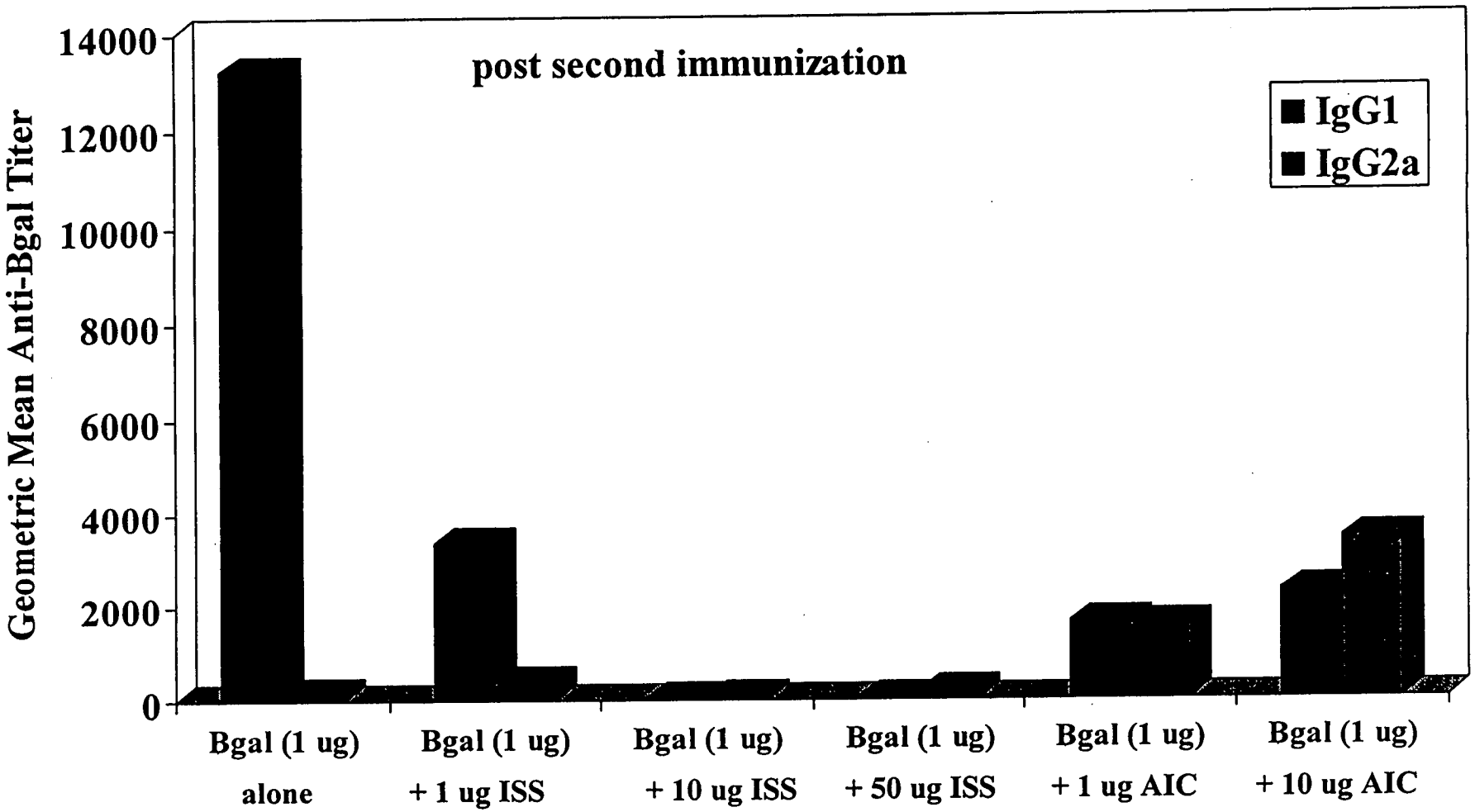
I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

October 22, 2002
Date

Gary Van Nest

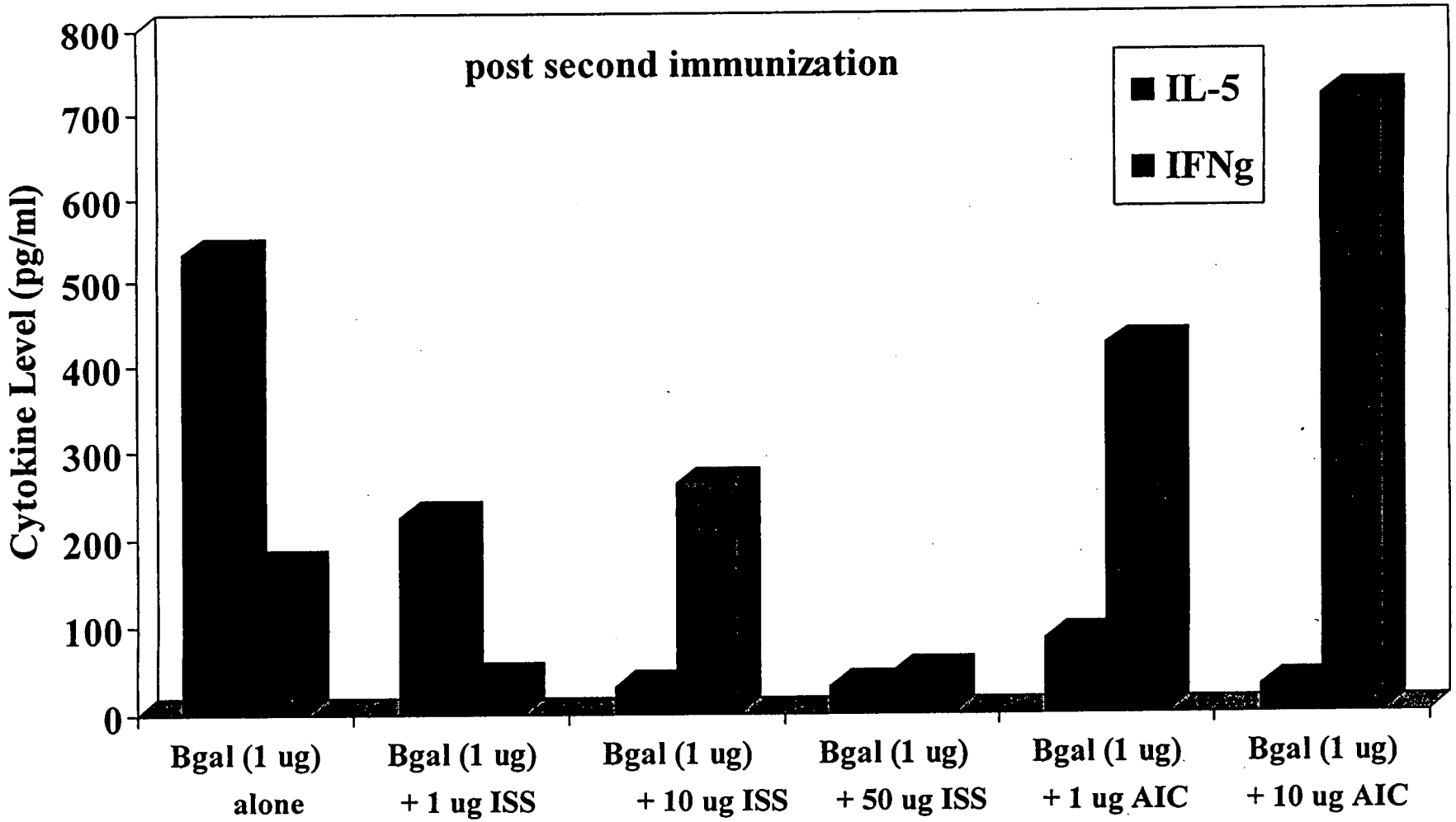
Anti-βgal Antibody Responses of Mice Co-administration of βgal with ISS or AIC

EXHIBIT A



Anti-βgal Cytokine Responses of Mice Co-administration of βgal with ISS or AIC

EXHIBIT B



A minimal human immunostimulatory CpG motif that potently induces IFN-γ and IFN-α production

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Recent reports have shown that immunostimulatory sequences (ISS) containing CpG motifs have minimal length requirements (≥12 bases) for the exertion of immune-enhancing function upon mammalian cells. Herein we demonstrate that short ISS (5–7 bases), which exhibit no activity on their own, induce IFN-γ and IFN-α secretion from human peripheral blood mononuclear cells when adsorbed to the surface of cationic poly(D,L-lactide-co-glycolide) microparticles (cPLGA). Utilizing this technique, we discovered a minimal ISS sequence for induction of IFN-γ and IFN-α from human cells: 5'-TCGXX-3'. These short ISS/cPLGA formulations targeted PDC in similar fashion to longer ISS ODN, the activity of which does not require (but is enhanced by) cPLGA. PDC stimulated with short ISS/cPLGA responded with enhanced uptake of ISS and elevated production of cytokines, including IFN-α. However, ISS-responsive B cells did not respond to short ISS/cPLGA, underlining the plasmacytoid dendritic cell selectivity of this formulation. These results describe a novel technique for formulating active, but very short, ISS oligodeoxynucleotide that allows for the dissection and characterization of minimal immunostimulatory CpG motifs.

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1 Introduction

Immunostimulatory sequence oligodeoxynucleotides (ISS ODN) are small pieces of DNA that contain an unmethylated cytosine-guanine dinucleotide within a particular sequence context (CpG motif). ISS ODN strongly stimulate the mammalian immune system by activating B cells, monocytes, and dendritic cells [1, 2], triggering the release of a variety of cytokines, and inducing potent antibody, Th1, and cytotoxic T lymphocyte (CTL) responses to antigens. The *in vivo* activity of ISS ODN as therapeutics in models of asthma and cancer and as vaccine adjuvants has been demonstrated in mice and primates [3–5]. Recently, the safety and efficacy of 1018 ISS was evaluated as a vaccine adjuvant in humans as well. The co-injection of 1018 ISS with hepatitis B surface antigen significantly enhanced the seroconversion rate and protective antibody response and was well tolerated by seronegative individuals [6].

Structure-activity studies have shown that certain oligonucleotides that have optimal immunostimulatory activity in mice can demonstrate relatively little activity in humans [7], although some sequences, such as 1018 ISS, have good activity in both species [8, 9]. The optimal consensus ISS motif for mice consists of a hexamer containing 5'-PuPuCGPyPy-3', where Pu is a purine and Py is a pyrimidine; however, the optimal ISS motif for humans has not been as clearly defined. ISS ODN that predominantly induce IFN-γ, IL-6, and B cell proliferation in human peripheral blood mononuclear cells (PBMC) generally consist of ODN with a fully modified phosphorothioate (PS) backbone and multiple CpG sequences. Various optimal human ISS motifs have been proposed, including GTCGTT [2, 10], TCGTT, and TCGTA [11]. Interestingly, ISS ODN containing these motifs described in the literature thus far generally do not induce significant levels of IFN-α from PBMC, although they perform other functions such as activation and proliferation of B cells and NK cells.

While the minimal mouse or human ISS motifs are most often described in the literature as hexameric se-

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Abbreviations: cPLGA: Cationic poly(D,L-lactide-co-glycolide) **FAM:** Fluorescein amidite **ISS:** Immunostimulatory sequence **MFI:** Mean fluorescence intensity **ODN:** Oligodeoxynucleotide **PDC:** Plasmacytoid dendritic cell **PO:** Phosphodiester **PS:** Phosphorothioate **Pu:** Purine **Py:** Pyrimidine

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quences, the bases outside of the hexamer also appear to be important for activity, although their exact role is unclear [12, 13]. Most ODN with significant activity in murine systems are 15–25 nucleotides in length, while shorter sequences lack activity [14]. In human PBMC, a length of >12 nucleotides is necessary to consistently induce immune responses such as B cell proliferation and the secretion of IL-6 and IgM, whereas a minimum length of about 14–16 nucleotides is required for detectable induction of IFN-γ [11, 15]. In fact, there has been no published report of ISS activity in human cells by ODN containing fewer than 8 nucleotides, and longer ODN are usually required for robust immune stimulation (Marshall, J. D., unpublished observations). Thus, the definition of the minimal human immunostimulatory motif is made difficult when explored in the context of large oligonucleotides, which require multiple CpG motifs and flanking sequences for significant activity.

Due to the relative maturity of both antisense ODN and DNA vaccine technologies, a large number of formulation and delivery strategies for DNA have been investigated. In the course of evaluating several of these strategies for ISS ODN delivery, we found that adsorption of ISS ODN to the surface of cationic poly(D,L-lactide-co-glycolide) microspheres (cPLGA) significantly enhanced the secretion of IFN-γ and IFN-α from human PBMC compared to ISS ODN alone. Previously, polymeric microspheres were shown to be a potent delivery system for DNA vaccines [16] and at least one CpG sequence [17]. Formulation of the ODN by this technique increases their uptake and stability, while also targeting the ISS to antigen-presenting cells [18]. We have used this formulation strategy as a tool to study the immunostimulatory activity of ODN containing <8 nucleotides, eliminating the need for flanking sequences to enhance uptake and allowing for the definition of a minimal immunostimulatory sequence required for induction of IFN-γ and IFN-α from human cells.

2 Results

2.1 Enhanced response of human PBMC to ISS formulated on cPLGA

1018 ISS (22-mer) and C274 (21-mer), each containing multiple CpG motifs and a phosphorothioate backbone, induced the secretion of IFN-γ and IFN-α from human PBMC. The IFN-γ response generated by C274 was somewhat higher than that induced by 1018 ISS, but more notably, C274 induced tenfold more IFN-α than 1018 ISS [19]. Others have reported that ISS-induced IFN-γ is NK cell-derived through an as yet undefined indirect mechanism, while IFN-α is induced directly by ISS ODN from plasmacytoid dendritic cells (PDC) [2]. We observed that the induction of these cytokines was magnified by the adsorption of the ISS ODN to the surface of cPLGA microspheres (Fig. 1). The specificity of sequence recognition was not altered by the formulation, as non-CpG negative control ODN 1040 + cPLGA demonstrated no cytokine induction.

2.2 Identification of the minimal human motif for IFN-γ/IFN-α induction by short ISS

In an effort to identify the minimal sequences that still retain the ability to induce enhanced levels of IFN-γ/IFN-α from PBMC, the 21-mer ISS ODN C274 sequence was divided and synthesized as three individual heptameric sequences: TCGTCGA, ACGTTCG, and AGATGAT. Both TCGTCGA and ACGTTCG contain two CpG dinucleotides, while AGATGAT does not contain a CpG sequence and served as a negative control. None of these heptameric CpG sequences was active in the human PBMC assay when used either alone or in combination up to a concentration of 100 μg/ml (data not shown). However, TCGTCGA induced substantial amounts of IFN-γ and IFN-α from human PBMC when formulated with cPLGA (Fig. 2A, B). Surprisingly, ACGTTCG, which also contains two CpG motifs, remained inactive even when formulated with cPLGA. These observations suggested a strategy for determining the optimum size, sequence, and position of the immunostimulatory motif without the confounding effects of flanking sequences that may be needed for cellular uptake or stability, but not for TLR9-mediated activation.

Comparison of the active and inactive heptamers from C274 suggested the hypothesis that TCG is required for

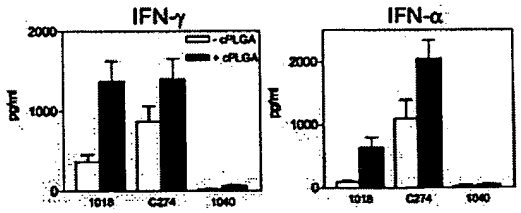

Fig. 1. cPLGA enhances the ability of ISS to stimulate IFN-γ/IFN-α production from PBMC. PBMC were stimulated for 24 h with 20 μg/ml ODN alone (white bars) or 20 μg/ml ODN complexed with 100 μg/ml cPLGA microparticles (gray bars). Data are reported as means of 27 separate donors + SEM. Statistical relevance: IFN γ: 1018 *vs.* 1040: ***; C274 *vs.* 1040: ***; 1018 *vs.* C274: *; 1018 *vs.* 1018 + cPLGA: ***; C274 *vs.* C274 + cPLGA: ns. IFN-α: 1018 *vs.* 1040: ns. C274 *vs.* 1040: **; 1018 *vs.* C274: **; 1018 *vs.* 1018 + cPLGA: **; C274 *vs.* C274 + cPLGA: *.

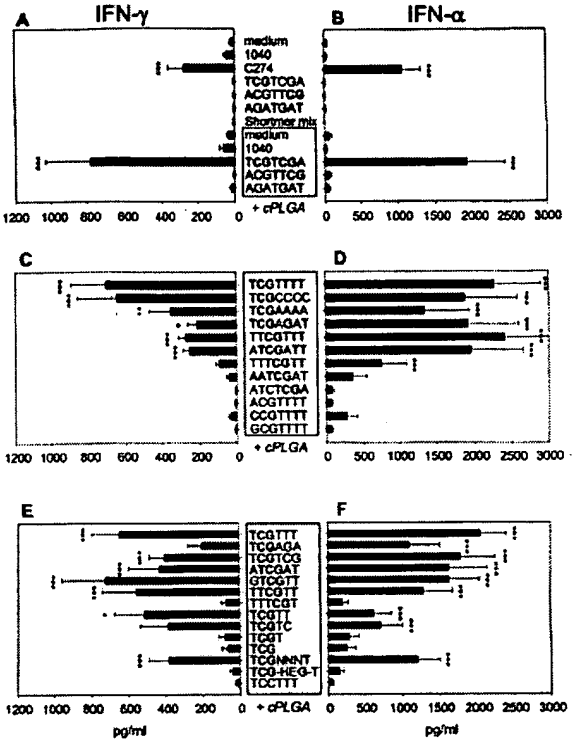

Fig. 2. Complexation with cPLGA confers potent ISS activity on short ISS ODN. PBMC were stimulated for 24 h with ISS ODN. Boxed ODN were complexed with cPLGA. Data are reported as means of 11–36 donors + SEM. Statistical relevance: each stimulatory condition compared to medium: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

human ISS activity and that the TCG motif must be located near the 5'-end (or distant to the 3'-end) of the short ISS. A series of heptamers, with a TCG motif placed in various positions throughout the sequence, was synthesized in order to study the importance of the TCG location. In addition, sequences containing ACG, CCG, and GCG were generated in order to determine the specific requirement for TCG in the immunostimulatory activity of the short ISS. As expected, none of the heptamers induced detectable IFN-γ or IFN-α in human PBMC when used as unformulated compounds (data not shown). However, for the heptamer ODN formulated with cPLGA, a TCG was absolutely necessary for immunostimulatory activity; heptamer sequences lacking a TCG (ACGTTTT, CCGTTTT, GCGTTTT) were uniformly inactive when formulated on cPLGA (Fig. 2C, D). In addition, the TCG was required to be either in the 5'-position (TCGTTTT, TCGCCCC, TCGAAAA, TCGAGAT) or the penultimate 5'-position (TTCGTTT, ATCGATT) in order to result in an optimally active ISS. Indeed, the immunostimulatory activity of the ODN was reduced as the TCG moiety was situated closer to the 3'-end (TTTCGTT, AATCGAT), until the oligonucleotides with the TCG in the penultimate 3'-position or 3'-position became completely inactive (ATCTCGA, ACGTTCG). The activity of the optimal heptameric ISS ODN adsorbed to cPLGA was comparable or superior to that observed with longer ISS ODN such as C274, which does not require cPLGA formulation for substantial IFN-γ/IFN-α activity (Fig. 2A, B).

Sequentially smaller ODN were synthesized in order to determine the minimum number of nucleotides necessary for immunostimulatory activity when adsorbed to cPLGA. A variety of hexameric and pentameric ODN were found to be active as long as they contained a TCG followed by at least two nucleotides on the 3'-end and were adsorbed to cPLGA (Fig. 2E, F). Conversely, the cPLGA-formulated trimer, 5'-TCG-3', and tetramer, 5'-TCGT-3', were inactive, further demonstrating the need for at least two nucleotides on the 3'-side of the TCG for immunostimulatory activity. Binding studies confirmed that the trimer and tetramer were adsorbed to the cPLGA at DNA loadings similar to longer ODN (data not shown); therefore, deficient binding to cPLGA was not the cause of the lack of activity of these compounds.

From the data generated above, the general human epitope was defined as 5'-TCGXX-3', where X is a nucleotide. The identity of X does not seem to be particularly important for immunostimulatory activity; rather, X appears to be necessary as a placeholder or binding site for the receptor. To further investigate this, two additional oligonucleotides were synthesized, each containing a 5'-TCG linked to a 3'-T by either three abasic (N) deoxyribose sugars (TCGNNNT) or a hexaethylene glycol (HEG) spacer (TCG-HEG-T) connected by phosphorothioate linkages. TCGNNNT/cPLGA induced IFN-γ and IFN-α from human PBMC in amounts similar to other 5'-TCG-containing heptamers formulated on cPLGA. In this case, the sugar/phosphate structure of the ODN was preserved, although there was no nucleobase present. Conversely, TCG-HEG-T/cPLGA was inactive in this assay, most likely due to the loss of the DNA-type structure in the position directly 3' to the TCG. Therefore, at least two additional residues that resemble nucleotides must be present 3' to the 5'-TCG motif for ISS activity.

2.3 Gene induction by short ISS formulated on cPLGA

To determine whether short ISS ODN/cPLGA complexes activate the immune system similarly to ISS ODN alone, the expression of an ISS-specific gene panel in human PBMC was evaluated after stimulation with C274, 1040, or a series of short ISS ODN formulated on cPLGA. The

Table 1. Short ISS/cPLGA induces similar pattern of gene expression to longer ISS[a]

Compound	2,5-OAS	ISG-54K	IFN-γ	IFN-α	IP-10	MIG	MCP-2
Medium	1	1	1	1	1	1	1
1040	1.1 (0.3)	1.1 (0.4)	1.5 (1.0)	22.5 (37.5)	2.0 (1.1)	1.2 (1.0)	0.9 (0.3)
C274	16.1 (9.5)	27.0 (12.6)	5.2 (2.5)	632.0 (402.1)	120.9 (131.8)	13.6 (11.8)	288.1 (396.0)
TCGTCGA	1.1 (0.4)	1.2 (0.5)	1.7 (0.2)	220.0 (377.7)	1.7 (0.2)	1.5 (0.6)	1.7 (0.9)
cPLGA	0.9 (0.1)	2.0 (0.4)	3.0 (1.5)	3.0 (2.9)	1.3 (0.1)	1.2 (0.7)	1.4 (1.0)
1040/cPLGA	2.6 (1.6)	2.3 (0.7)	3.8 (2.5)	61.1 (49.1)	1.9 (1.3)	1.7 (1.4)	3.3 (2.8)
TCGTCGA/cPLGA	14.3 (2.8)	20.6 (7.9)	36.4 (50.9	1317.4 (1436.5)	49.0 (54.8)	19.3 (24.9)	126.9 (122.3)
TCGTTTT/cPLGA	20.1 (13.8)	25.2 (8.2)	66.2 (57.5)	1099.4 (722.0)	82.3 (61.5)	32.5 (26.5)	349.7 (370.9)
TCGTTT/cPLGA	21.5 (20.2)	18.6 (7.9)	32.3 (27.9)	583.3 (478.4)	63.9 (55.9)	20.0 (20.7)	242.9 (326.4)
TTCGTT/cPLGA	17.4 (16.4)	19.4 (6.7)	25.2 (23.0)	414.8 (229.0)	33.4 (28.9)	21.8 (18.7)	200.6 (223.4)
TCCTTT/cPLGA	1.7 (2.1)	2.3 (1.5)	4.1 (3.7)	1.7 (0.2)	1.7 (2.1)	1.3 (1.4)	3.9 (5.4)

[a] PBMC from four donors were cultured for 10 h with 5 μg/ml short ISS ± PLGA and RNA was extracted and quantitated via TaqMan RT-PCR. Data are presented as the mean of fold induction over medium control (given the value of 1.0) with SEM. A second experiment using four donors gave similar results.

RNA was harvested at 10 h, converted to cDNA, and evaluated by quantitative PCR. The expression levels of each gene were normalized to ubiquitin signal and then calculated as fold-increases over medium stimulation. This ISS-specific gene panel was previously identified in our laboratory as a sensitive marker for ISS activity in human PBMC [19]. Table 1 shows that C274 and the most potent cPLGA-formulated short ODN containing the optimal ISS motif (TCGTCGA, TCGTTTT, TCGTTT, TTCGTT) induced high levels of mRNA for the cytokines IFN-γ and IFN-α, the IFN-α-inducible genes, 2,5-oligoadenylate synthetase (2,5-OAS) and interferon-stimulated gene-54K (ISG-54K), and the chemokines interferon-inducible protein-10 (IP-10), monokine induced by IFN-γ (MIG), and monocyte chemoattractant protein-2 (MCP-2). The control ODN 1040, alone or formulated on cPLGA, had little effect on gene expression. As expected, the unformulated heptamer, TCGTCGA, and the formulated short control ODN, TCCTTT, also did not significantly alter mRNA levels. These data suggest that short ISS + cPLGA transmits similar signals as standard ISS which result in comparable patterns of gene activation.

2.4 Cellular targets of short ISS formulated on cPLGA

To verify that ISS ODN and short ISS/cPLGA complexes are active on the same cell types, PDC were isolated and purified from human PBMC by immunomagnetic bead positive selection using BCDA-4. We and others have previously established that human PDC can respond to ISS stimulation with the secretion of IFN-α and TNF-α [2, 20] and that C274 is particularly potent in this respect [19]. PDC were stimulated with C274, 1040, or a series of short ISS/cPLGA complexes for 24 h, after which SN were harvested and analyzed for cytokine content. As expected, C274 induced the secretion of high levels of both IFN-α and TNF-α from PDC, while the control ODN 1040 had no effect (Fig. 3). Short ISS ODN/cPLGA complexes with the sequences TCGTCGA and TCGTTTT also directly activated the PDC to produce IFN-α and TNF-α. These same short ISS/cPLGA complexes also induced elevated IFN-α gene expression at 4 h from the PDC, independently confirming the ELISA data (data not shown).

ISS ODN are also known to directly activate a subset of B cells, causing them to proliferate and secrete IL-6. These activities were measured for purified peripheral blood B cells, which had been stimulated with a similar panel of short ISS/cPLGA complexes (Fig. 3). As expected, C274 stimulated B cells to both proliferate and release IL-6. 1040 induced minimal levels of activity in both assays, consistent with previous observations that all PS ODN can marginally activate B cells through a CpG-irrelevant pathway [21]. In contrast, the short ISS/cPLGA formulations were inert on B cells (Fig. 3), indicating that this type of complex specifically targets the PDC arm of the ISS response. Interestingly, longer ISS, such as C274, when complexed on cPLGA, are recognized by B cells, which respond with activity equivalent to that induced by the longer ISS alone (data not shown), indicating a fundamental distinction between how B cells recognize short vs. long ISS displayed on cPLGA.

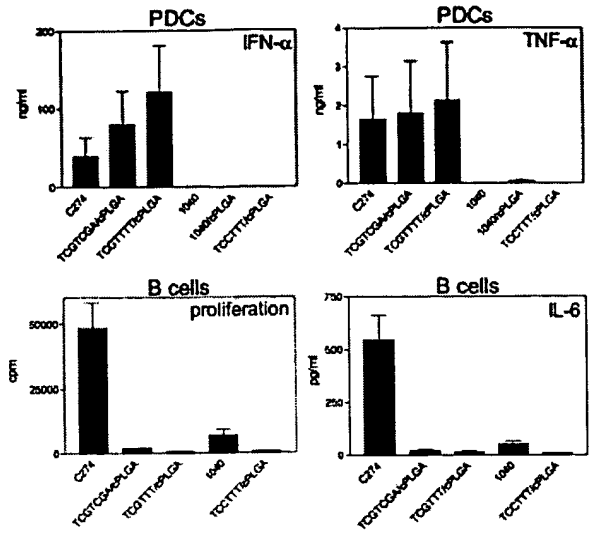

Fig. 3. Short ISS/cPLGA exerts ISS activity on PDC but is inert on B cells. MACS-purified PDC were cultured with 5 μg/ml ISS ± cPLGA for 24 h. MACS-purified B cells were cultured with 5 μg/ml ISS ± cPLGA for 72 h (proliferation) or 48 h (IL 6). Proliferation was assessed by [^{3}H]thymidine incorporation and IL-6 by ELISA. Data are reported as the means of four donors + SEM.

2.5 cPLGA enhances uptake of short ISS into PDC

Previous reports have shown that cellular uptake of ODN is sequence-independent and saturable [22]. Using ODN with a 3'-fluorescein label (FAM-ODN), we observed CpG motif-independent uptake of ODN by human monocytes, B cells, myeloid dendritic cells, and PDC (data not shown). Further studies were focused on DNA uptake by PDC within a monocyte-depleted PBMC population, because PDC are directly activated by ISS ODN while monocytes are functionally unresponsive to direct ISS stimulation yet act as a large ODN sink that can obscure ISS uptake by the much smaller PDC population. Short ODN (TCGTCGA and AGATGAT) were minimally taken up by PDC compared to longer ODN (C274 and 1040, Table 2). However, adsorption of long or short ODN on the surface of cPLGA microparticles significantly increased the number of PDC taking up FAM-ODN (% double positive for fluorescein and BCDA-4), as well as the amount of ODN taken up per cell (MFI). Short ISS formulated with cationic liposomes, another known method of increasing DNA uptake into cells [23], also yielded similar uptake and activity results in comparison to cPLGA formulations (data not shown). This suggests that greatly enhancing the uptake of short ISS into PDC through adsorption to cPLGA is responsible for the substantially increased activity of short ISS/cPLGA.

Table 2. Uptake of short ISS is enhanced by formulation with cPLGA or liposomes[a]

ODN	% ODN-FAM positive[b]	MFI[c]
Medium	1.7 (1.6)	2 (<1)
C274	90.0 (5.5)	12 (2)
1040	68.0 (16.6)	8 (2)
TCGTCGA	4.8 (2.0)	3 (1)
AGATGAT	3.4 (0.9)	2 (<1)
cPLGA	1.6 (1.7)	2 (<1)
C274/cPLGA	94.3 (3.7)	72 (43)
1040/cPLGA	93.9 (4.6)	272 (137)
TCGTCGA/cPLGA	88.6 (7.8)	147 (118)
AGATGAT/cPLGA	91.5 (6.1)	325 (188)

[a] Monocyte-depleted PBMC were incubated with FAM-labeled ODN alone or complexed with cPLGA for 30 min. Cells were then stained with BCDA-4-PE and analyzed via FACS for intracellular FAM-ISS content. Data are reported as the means (SD) of four donors. These data are from one of three representative experiments.

[b] % of BCDA-4$^+$ PDC that were positive for ODN-FAM.

[c] MFI, mean fluorescent intensity.

2.6 Short ISS ODN compete for recognition with longer ISS

If short ISS ODN bind to the same receptor that recognizes standard ISS (thought to be TLR9), then they should be able to compete with longer ISS ODN when present in excess. To examine this question, we performed ODN competition studies in which a dose titration of C274 (0.3–20 μg/ml) was conducted in the presence or absence of an excess of short ISS ODN. Since previous experiments had shown that short ISS were taken up less efficiently than the molar equivalent of longer ODN (Table 2), we used 50 μg/ml short ISS in the competition study, a concentration which we found allowed uptake into PDC comparable to tenfold lower concentrations of longer ISS (data not shown). Fig. 4 shows that an excess of either TCGTCGA or the longer ISS ODN 1018 successfully suppressed the ability of C274 to induce IFN-α from PBMC, while the short ISS ODN, ACGTTCG and AGATGAT, which lack activity even when complexed with cPLGA, did not. FACS analysis with propidium iodide indicated that excess TCGTCGA did not exhibit any toxic effects on the cells (data not shown). This suggests that TCGTCGA ODN recognize the same receptor as standard ISS but may have much

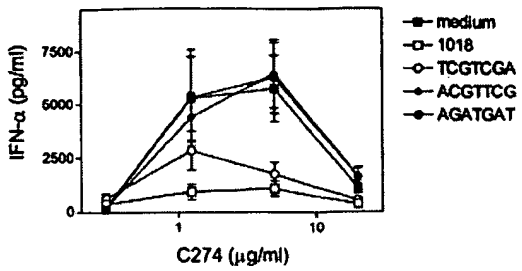

Fig. 4. Excess short ISS competes away activity of C274. PBMC were stimulated for 24 h with C274 (concentration range: 20, 5, 1.25, 0.3 μg/ml) in the absence or presence of 50 μg/ml 1018 or short ISS. Data are reported as means of ten separate donors + SEM.

lower affinity and weaker signaling power, since highly increased uptake via formulation is required for them to exert IFN-γ/α-inducing activity.

3 Discussion

Although an unmethylated CpG dinucleotide is required for biological activity, it has been shown that specific flanking bases are required for optimal activity [11, 24]. Although consensus exists for the optimal recognition motif for mouse activity (PuPuCGPyPy) [25], a comparable human motif has not yet been clearly defined. The sequence(s) recognized optimally by human TLR9 is likely similar in size to the hexameric mouse motif, but definition has been complicated by the fact that active oligonucleotides must be at least 12–15 bases long [14] and require at least two CpG motifs [10] to exhibit clear ISS activity *in vitro*. In this report, we show that complexing active ISS ODN to cPLGA microparticles significantly enhances their ability to induce IFN-γ and IFN-α production from PBMC. The specificity of ISS ODN recognition is not altered, as molecules lacking active CpG motifs remain inactive when complexed to cPLGA. The fact that ISS ODN complexed to cPLGA are not constrained by sequence length has allowed us to discover a motif of minimal length (5'-TCGXX-3') that potently stimulates for interferon production.

As has been previously reported, the optimal recognition motif for human PBMC includes a CpG dinucleotide preceded by T, rather than by two purine bases, as is the case for mouse cells [2, 10]. However, we found that the simple presence of a TCG within a short ISS sequence was not sufficient to confer activity when in cPLGA-complexed form. In fact, the position of the TCG motif was required to be at or very near the 5' end of the short ODN, allowing at least two additional bases 3' to the TCG. Sequence variation studies showed that ODN activity diminished as the TCG motif was moved farther from the 5' end and closer to the 3' end (Fig. 2). This principle is demonstrated by the inactivity of the sequence ACGTTCG, which, despite containing two CpG (one of which is a TCG), failed to meet the requirements of having the TCG near the 5' end and at least two bases on the 3' end of the TCG. Requirement for positioning of the CpG motif at the 5' end of short ISS was also observed by Tidd et al. [26], although that study examined intracytoplasmic delivery of short ISS to induce apoptosis in the MOLT-4 T cell leukemic line. We further discovered that the required 3' bases could be of any combination and could even be abasic sugar residues. This suggests that at least two nucleotide lengths of sequence nonspecific DNA are required to be present 3' of the motif, perhaps to stabilize the interaction with the TLR9 receptor. It is interesting to note that the rules we observed governing the ability of short ISS/cPLGA to stimulate optimally for IFN-α production may not fully explain the high IFN-α induction by certain longer ISS ODN, such as C274 [19]. Other sequences containing 5'-TCG, such as 2006 (TCGTCGTTTTGTCGTTTTGTCGTT), have been well documented as poor IFN-α-inducers [27]. Thus, 5'-TCG appears to be necessary but not always sufficient for high IFN-α induction by ISS of standard length.

Our data show that short heptameric ISS ODN are not taken up by PDC as efficiently as longer ODN of approximately 20 bases. However, formulating short ISS with agents that promote DNA uptake (cPLGA or liposomes) confers activity upon 5'-TCG-containing heptameric ODN. Sonehara et al. [28] described that CpG-containing palindromic hexamers were inactive on their own but were converted to active form when formulated with cationic liposomes. However, these studies were conducted with PO, not PS, ODN and ISS activity was measured as mouse splenocyte production of interferons. We have observed that short PO ISS are inactive on human cells when formulated with liposomes (data not shown), presumably due to enzymatic degradation, but are protected when complexed with cPLGA and retain ISS activity. These observations suggest a difference in nuclease production between human and mouse cells [7]. In addition, we found that both the PO and PS versions of the optimal motif (AACGTT) described by Sonehara et al. [28] as exhibiting mouse activity demonstrated no such activity on human cells, even when cPLGA- or liposome-formulated (data not shown). This suggests further mouse *vs.* human differences, *i.e.* in the specificity by which their ISS receptors recognize optimal ISS motifs.

This laboratory has found that ISS molecules such as C274 trigger a pattern of gene activation from human PBMC that includes up-regulation of message levels for

IFN-γ and IFN-α, the chemokines IP-10, MIG, and MCP-2, and the IFN-α-inducible genes 2,5-OAS and ISG-54K [19]. The finding that short ISS + cPLGA induced the same pattern of gene activation as the longer active ISS ODN demonstrates that these two types of ISS deliver comparable signals to responsive cell populations.

Although readily recognized by PDC, short ISS ODN/cPLGA complexes did not induce detectable ISS responses from B cells, despite enhancement of uptake into B cells when cPLGA-associated (data not shown). A similar disparity in the ISS response between responder cell types has been reported when comparing two major classes of ISS ODN, known variously as CpG-A and CpG-B [29]. ODN of the first type (CpG-A) induce large amounts of IFN-α from PDC and IFN-γ from NK cells but are inert on B cells [11], while ODN of the second type (CpG-B) promote B cell functions but are relatively poor for IFN-γ/IFN-α production [2]. A third and newly described class of ISS ODN, termed CpG-C and including C274, is able to induce strong ISS responses from both cell types [19]. The short ISS ODN/cPLGA complexes do not fit the chemical or sequence definitions for CpG-A ODN (PO/PS chimeric DNA backbone, polyguanosine motif), despite their similar profile of ISS activities. However, these results may indicate that multimeric presentation of ISS motifs, either by adsorption to an insoluble support for short ISS or through aggregation for CpG-A [21], preferentially amplifies IFN-α secretion from PDC over other ISS functions. These findings indicate that the motif, length, and/or presentation requirements for IFN-γ/α production may be different from the requirements for B cell activation, which in turn suggests a fundamental difference in the manner in which these two cell types recognize ISS. This may be due to differential ISS receptors between B cells and PDC, which might be represented by alternately spliced variants of TLR9, by TLR9 heterodimerized with other TLR family members, by unidentified non-TLR9 ISS receptors, or by differential intracellular compartmentalization. Although not recognized by B cells, the facts that short ISS/cPLGA complexes signal very similarly to standard ISS on PDC and that short ISS can compete away longer ISS activity suggests that short ISS interacts with the same ISS receptor as longer ODN in PDC.

In conclusion, these studies define a minimal ISS motif for the optimal induction of IFN-γ and IFN-α from human PBMC to be 5'-TCGXX-3', where X can be any nucleotide. The element of 5'-TCG has also been noted as crucial for high IFN-α induction by longer ISS as well, although other elements such as the presence of a palindrome, its length, and incorporation of other CpG motifs are also necessary [19]. We have not determined whether this is the minimal motif required for other ISS functions such as dendritic cell maturation and differentiation, and it may be that other immunomodulatory motifs also exist that target those functions. Additionally, we have described a system of formulation that can dramatically enhance the function of any ISS molecule, even those ISS ODN too short to have activity on their own. Since cPLGA microparticles are biodegradable within living systems, this formulation might serve as a potent adjuvant for ISS-based therapeutics.

4 Materials and methods

4.1 Oligodeoxynucleotides

Phosphorothioate ODN were prepared on an Expedite 8909 following manufacturers protocols, purified by RP-HPLC, and precipitated as the sodium salt. ODN sequences, here and throughout, are listed 5' to 3'. 1018: TGACTGTGA-ACGTTCGAGATGA; 1040: TGACTGTGAACCTTAGA-GATGA; C274: TCGTCGAACGTTCGAGATGAT. 3'-Fluorescein-labeled ODN were prepared using 6-FAM-CPG (Glen Research). All ODN had <5 endotoxin units/mg of ODN as determined by Limulus amebocyte lysate assay (BioWhittaker).

4.2 Preparation of cPLGA microspheres

cPLGA (0.875 g, Resomer® RG502, Boehringer Ingelheim Chemicals) and 1,2-dioleoyl-3-trimethylammoniumpropane (0.3 g, Avanti Polar Lipids) were dissolved in methylene chloride (7.9 g) as described [16]. The cPLGA was characterized for size and surface charge: mean size (number weighted, μ) =1.4; zeta potential (mV) =32.4.

4.3 Preparation of ODN/cPLGA complexes

The ODN and cPLGA were mixed at final concentrations of 5–20 μg/ml and 100 μg/ml, respectively, for 15 min at room temperature before they were added to the culture. These concentrations were derived previously as optimal for the induction of PBMC IFN-γ.

4.4 Mononuclear cell preparation

Human PBMC were isolated as described [19] and cultured in RPMI 1640 (BioWhittaker) supplemented with 10% heat-inactivated human AB serum (Gemini) plus 50 U/ml penicillin, 50 μg/ml streptomycin, 300 μg/ml glutamine, 1 mM sodium pyruvate (BioWhittaker), and 1× nonessential amino acids (BioWhittaker). For cytokine secretion, PBMC were cultured at 0.5×10^6/well in 96-well flat-bottom plates in duplicate with ISS ODN at 20 μg/ml ± cPLGA for 24 h, determined by previous studies to be the optimal time point for ISS-induced cytokine secretion. In the competitive receptor

binding experiment, a titrated dose range of C274 (20, 5, 1.25, and 0.3 μg/ml) was premixed with 50 μg/ml of short ODN for 15 min at room temperature in media, then immediately added to culture so that the PBMC were exposed to both types of ODN simultaneously. Cell-free SN were harvested and assayed by ELISA, as described [19].

4.5 Statistical analysis

Statistical significance was calculated using an unpaired *t*-test with Welch correction, assuming parametric data with different S.D.'s, to get two-tailed *p* values (GraphPad InStat). Symbols representing significance are: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; ns, $p>0.05$.

4.6 PDC and B cell purification and functional assays

Human PDC and B cells were isolated as described [19]. For cytokine secretion, PDC were cultured at 0.5×10^5–1×10^5/well (2×10^5–4×10^5/ml) in 96-well round-bottom plates with 5 μg/ml ISS ± cPLGA for 24 h, then SN were harvested and assayed for cytokines via ELISA. For the proliferation assay, B cells were cultured in triplicate at 1×10^5/well in 96-well round-bottom plates with 2 μg/ml ODN ± cPLGA for 72 h. At the end of the culture period, the plates were pulsed with [^{3}H]thymidine (1 μCi/well, Amersham) and incubated for an additional 8 h. Then the plates were harvested and radioactive incorporation determined using standard liquid scintillation techniques, and the data was collected in counts per minute (cpm). For IL-6 secretion, B cells were cultured at 0.5×10^6–1×10^6/well in 48-well plates with 5 μg/ml ISS ± cPLGA for 48 h, then SN were harvested and assayed for IL-6 via ELISA.

4.7 Gene expression assay and analysis

Human PBMC were cultured with ISS ± cPLGA at 2×10^6/ml for 10 h, then RNA extracted and analyzed via TaqMan RT-PCR as described [19]. Primer sequences for ubiquitin, 2,5-OAS, ISG-54 K, MIG, MCP-2, and IFN-α were synthesized by Operon and are referenced in [19]. IFN-γ and IP-10 were measured using PDAR supplied by Applied BioSystems. Threshold cycle (C_T) values for each gene were normalized to ubiquitin. The negative control for each experiment, stimulation with medium alone, was assigned a value of 1 and all data expressed as fold induction over the negative control.

4.8 DNA uptake assay

Human PBMC were depleted of monocytes by positive section with CD14-MACS beads [30]. The resultant population was cultured at 2×10^6/ml for 2 h with FAM-labeled ODN: 5 μg/ml C274 and 1040 and 1.67 μg/ml TCGTCGA and AGATGAT (to keep molar equivalence) ± cPLGA. Cells were harvested and washed three times with ice-cold 1% BSA-PBS to remove surface-associated ODN, then stained with BCDA-4-PE [30] and analyzed via FACScan. Propidium iodide staining was used to gate on living cells. Data are reported as % FAM-positive (ISS$^+$) cells within the BCDA-4$^+$ population and the MFI is also derived from that group. Liposome/ODN complexes were formulated with GenePORTER 2 Transfection Reagent (Gene Therapy Systems) according to the manufacturer's protocol.

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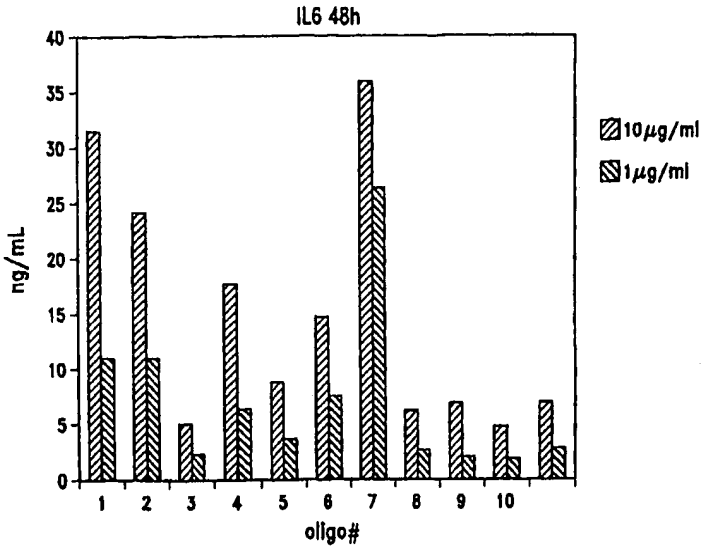

(57) Abstract

The invention relates to immunostimulatory oligonucleotide compositions. These oligonucleotides comprise an immunostimulatory octanucleotide sequence. These oligonucleotides can be administered in conjunction with an immunostimulatory peptide or antigen. Methods for modulating an immune response upon administration of the oligonucleotide are also disclosed. In addition, an in vitro screening method to identify oligonucleotides with immunostimulatory activity is provided.

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IMMUNOSTIMULATORY OLIGONUCLEOTIDES, COMPOSITIONS THEREOF AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the priority benefit of U.S. provisional patent application No. 60/048,793 filed June 6, 1997, pending. The aforementioned provisional application is hereby incorporated herein by reference in its entirety.

TECHNICAL FIELD

The present invention relates to immunomodulatory compositions comprising an immunostimulatory oligonucleotide sequence (ISS). The invention further relates to immunomodulatory compositions comprising an ISS in which at least one base has been substituted with a base modified by the addition to C-5 or C-6 on cytosine with an electron-withdrawing moiety. It also relates to the administration of the oligonucleotide sequences to modulate at least one immune response. The invention further relates to *in vitro* screening methods to identify oligonucleotides with potential immunomodulatory activity.

BACKGROUND ART

The type of immune response generated to infection or other antigenic challenge can generally be distinguished by the subset of T helper (Th) cells involved in the response. The Th1 subset is responsible for classical cell-mediated functions such as delayed-type hypersensitivity and activation of cytotoxic T lymphocytes (CTLs), whereas the Th2 subset functions more effectively as a helper for B-cell activation. The type of immune response to an antigen is generally determined by the cytokines produced by the cells responding to the antigen. Differences in the cytokines secreted by Th1 and Th2 cells are believed to reflect different biological functions of these two subsets.

The Th1 subset may be particularly suited to respond to viral infections and intracellular pathogens because it secretes IL-2 and IFN-γ, which activate CTLs. The Th2 subset may be more suited to respond to free-living bacteria and helminthic parasites and may mediate allergic reactions, since IL-4 and IL-5 are known to induce IgE production and eosinophil activation, respectively. In general, Th1 and Th2 cells secrete distinct patterns of cytokines and so one type of response can moderate the activity of the other type of response. A shift in the Th1/Th2 balance can result in an allergic response, for example, or, alternatively, in an increased CTL response.

Immunization of a host animal against a particular antigen has been accomplished traditionally by repeatedly vaccinating the host with an immunogenic form of the antigen. While most current vaccines elicit effective humoral (antibody, or "Th2-type") responses, they fail to elicit cellular responses (in particular, major histocompatibility complex (MHC) class I-restricted CTL, or "Th1-type" responses) which are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, Th2-type responses are of little protective value against infection. Moreover, antibody responses are inappropriate in certain indications, most notably in allergy where

an antibody response can result in anaphylactic shock. Proposed vaccines using small peptides derived from the target antigen and other currently used antigenic agents that avoid use of potentially infective intact viral particles, do not always elicit the immune response necessary to achieve a therapeutic effect. The lack of a therapeutically effective human immunodeficiency virus (HIV) vaccine is an unfortunate example of this failure.

Protein-based vaccines typically induce Th2-type immune responses, characterized by high titers of neutralizing antibodies but without significant cell-mediated immunity. In contrast, intradermal delivery of "naked", or uncomplexed, DNA encoding an antigen stimulates immune responses to the antigen with a Th1-type bias, characterized by the expansion of $CD4^+$ T cells producing IFN-γ and cytotoxic $CD8^+$ T cells. Manickan et al. (1995) *J. Immunol.* 155:250-265; Xiang et al. (1995) *Immunity* 2:129-135; Raz et al. (1995) *Proc. Natl. Acad. Sci. USA* 93:5141-5145; and Briode et al. (1997) *J. Allergy Clin. Immunol.* 99:s129. Injection of antigen-encoding naked DNA reproducibly induces both humoral and cellular immune responses against the encoded antigens. Pardoll and Beckerleg (1995) *Immunity* 3:165-169. DNA vaccines can provide a new approach to infectious disease prophylaxis. See, for instance, Dixon (1995) *Bio/Technology* 13:420 and references cited therein.

Certain types of DNA, without being translated, have been shown to stimulate immune responses. Bacterial DNA induces anti-DNA antibodies in injected mice, as well as cytokine production by macrophage and natural killer (NK) cells. Pisetsky (1996) *J. Immunol.* 156:421-423; Shimada et al. (1986) *Jpn. J. Cancer Res.* 77:808-816; Yamamoto et al. (1992a) *Microbiol. Immunol.* 36:983-897; and Cowdery et al. (1996) *J. Immunol.* 156:4570-4575.

B cell and NK cell activation properties of bacterial DNA have been associated with short (6 base pair hexamer) sequences that include a central unmethylated CpG dinucleotide. Yamamoto et al. (1992a); and Krieg et al. (1995) *Nature* 374:546-549. Oligonucleotides comprising a CpG sequence flanked by two 5' purines and two 3' pyrimidines have been shown to be most potent in B cell and NK cell stimulation. For example, when a variety of oligonucleotides comprising hexamers were tested for their ability to augment the NK cell activity of mouse spleen cells, the most immunogenic hexamers included AACGTT, AGCGCT, GACGTC. Yamamoto et al. (1992b) *J. Immunol.* 148:4072-4076. In a study in which B cell activation was measured in response to oligonucleotides, the most stimulatory hexamer sequences (e.g., AACGTC, AACGTT, GACGTC, GACGTT) also matched the sequence of 5'-purine, purine, CG, pyrimidine, pyrimidine-3'. Krieg et al. (1995). However, as shown herein, this prototypical hexamer sequence is found in many oligonucleotides that are not immunostimulatory. Thus, the prototypical hexamer sequence proposed by Krieg et al. (1995) is not predictive of immunostimulatory activity.

Bacterial DNA stimulated macrophages to produce IL-12 and TNF-α. These macrophage-produced cytokines were found to induce the production of IL-12 and IFN-γ from splenocytes. Halpern et al. (1996) *Cell. Immunol.* 167:72-78. *In vitro* treatment of splenocytes with either bacterial DNA or CpG containing oligonucleotides induced the production of IL-6, IL-12 and IFN-γ. Klinman et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:2879-2883. Production of all of these cytokines is indicative of induction of a Th1-type immune response rather than a Th2-type response.

To date, no clear consensus has been reached on the sequences both necessary and sufficient of immune stimulation. A recent study which examined induction of NK activity in response to CpG containing-oligonucleotides suggested that the unmethylated CpG motif was necessary but not sufficient for oligonucleotide induction of NK lytic activity. Ballas et al. (1996) *J. Immunol.* 157:1840-1845. Sequences flanking the CpG appeared to influence the immunostimulatory activity of an oligonucleotide. Immunostimulatory activity of immunostimulatory sequences appears to be independent of adenosine-methylation, and whether the nucleotide is single or double-stranded. See, for example, Tokunaga et al. (1989) *Microbiol. Immunol.* 33:929; Tokunaga et al. (1992) *Microbiol. Immunol.* 36:55-66; Yamamoto et al. (1992b); Messina et al. (1993) *Cell. Immunol.* 147:148-157; and Sato et al. (1996) *Science* 273:352-354. Oligonucleotide length also does not seem to be a factor, as double-stranded DNA 4 kb long (Sato et al. (1996)) or single-stranded DNA as short as 15 nucleotides in length (Ballas et al. (1996)) illicited immune responses; though if oligonucleotide length was reduced below 8 bases or if the DNA was methylated with CpG methylase, immunostimulatory activity was abolished. Krieg et al. (1995).

Allergic responses, including those of allergic asthma, are characterized by an early phase response, which occurs within seconds to minutes of allergen exposure and is characterized by cellular degranulation, and a late phase response, which occurs 4 to 24 hours later and is characterized by infiltration of eosinophils into the site of allergen exposure. Specifically, during the early phase of the allergic response, activation of Th2-type lymphocytes stimulates the production of antigen-specific IgE antibodies, which in turn triggers the release of histamine and other mediators of inflammation from mast cells and basophils. During the late phase response, IL-4 and IL-5 production by $CD4^+$ Th2 cells is elevated. These cytokines appear to play a significant role in recruiting eosinophils into site of alleregen exposure, where tissue damage and dysfunction result.

Antigen immunotherapy for allergic disorders involves the subcutaneous injection of small, but gradually increasing amounts, of antigen. Such immunization treatments present the risk of inducing IgE-mediated anaphylaxis and do not address the cytokine-mediated events of the allergic late phase response.

Vaccination with certain DNA containing immunostimulatory motifs induces an immune response with a Th1-type bias. For example, mice injected intradermally with *Escherichia coli (E. coli)* β-galactosidase (β-Gal) in saline or in the adjuvant alum responded by producing specific IgG1 and IgE antibodies, and $CD4^+$ cells that secreted IL-4 and IL-5, but not IFN-γ, demonstrating that the T cells were predominantly of the Th2 subset. However, mice injected intradermally (or with a tyne skin scratch applicator) with plasmid DNA (in saline) encoding β-Gal and containing an ISS responded by producing IgG2a antibodies and $CD4^+$ cells that secreted IFN-γ, but not IL-4 and IL-5, demonstrating that the T cells were predominantly of the Th1 subset. Moreover, specific IgE production by the plasmid DNA-injected mice was reduced 66-75%. Raz et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:5141-5145. In general, the response to naked DNA immunization is characterized by production of IL-2, TNFα and IFN-γ by antigen-stimulated $CD4^+$ T cells, which is indicative of a Th1-type response. This is particularly important in treatment of allergy and asthma as shown by the decreased IgE production.

In another example, the presence of an immunstimulatory sequence, such as the palindromic hexamer AACGTT, in an antigen-encoding plasmid vector injected intradermally prompted the production of large amounts of IFN-α, IFN-β and IL-12. Sato et al. (1996). IFN-α plays a role in the differentiation of naive T cells toward a Th1-type phenotype, antagonizes Th2 cells, inhibits IgE synthesis, promotes IgG2a production and induces a Th1 phenotype of antigen-specific T cell clones. IL-12 promotes IFN-γ production by T cells and favors maturation of Th1 cells.

It would be useful in treatment of a wide variety of indications to be able to specifically enhance the Th1-type response to a particular antigen while down-regulating the Th2-type respone to the same antigen. Treatment or palliation of these indications includes, but is not limited to, tumor therapy, treatment of allergic disorders and induction of a vigorous cellular immune response. The present invention provides compositions comprising oligonucleotide sequences that can be employed in these contexts.

All of the cited literature included in the preceding section, as well as the cited literature included in the following disclosure, are hereby incorporated herein by reference.

DISCLOSURE OF THE INVENTION

The present invention provides immunomodulatory compositions comprising an oligonucleotide that contains at least one immunostimulatory (ISS) octanucleotide.

In a preferred embodiment, the ISS octanucleotide comprises the sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, Cytosine-3'.

In another preferred embodiment, the ISS octanucleotide comprises the sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, Guanine-3'.

In a further embodiment, the ISS octanucleotide is selected from AACGTTCC, AACGTTCG, GACGTTCC and GACGTTCG.

In another embodiment, at least one of the cytosines of the ISS octanucleotide sequence is substituted with a modified cytosine, wherein the modified cytosine comprises an addition of an electron-withdrawing group to at least C-5 and/or C-6. Preferably, the modified cytosine is 5'-bromocytidine. Preferably, the C at the third position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine.

In another embodiment, the immunomodulatory composition comprises an oligonucleotide that contains at least one ISS octanucleotide and an antigen.

In a further embodiment, the antigen is selected from the group consisting of proteins, glycoproteins, polysaccharides, and lipids.

In another embodiment, the antigen is conjugated to the ISS oligonucleotide.

In another embodiment, the immunomodulatory composition comprises an oligonucleotide that contains at least one immunostimulatory (ISS) octanucleotide and a facilitator selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligand, a trans-activating factor, a peptide, and a peptide comprising a modified amino acid.

In another embodiment, the immunomodulatory composition comprises an oligonucleotide that contains at least one ISS octanucleotide, an antigen, and an adjuvant.

In another embodiment, an immunomodulatory composition comprises an immunomodulatory oligonucleotide and an antigen proximately associated at a distance effective to enhance an immune response.

In another embodiment, an immunomodulatory composition comprises an immunomodulatory oligonucleotide and an antigen proximately associated to co-deliver the oligonucleotide and the antigen to an immune target.

In another embodiment, an immunomodulatory composition comprises an immunomodulatory oligonucleotide and the antigen associated with an adjuvant. Further, the immunomodulatory oligonucleotide and the antigen are associated in microparticles. In another embodiment, the immunomodulatory oligonucleotide and the antigen are associated in liposomes.

The invention also provides for methods of modulating an immune response comprising the administration of an immunomodulatory composition comprising an antigen and an oligonucleotide that contains at least one ISS octanucleotide.

In a further embodiment, the immune response modulation comprises the induction of a Th1 response.

The invention also provides for a method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory facilitator and an oligonucleotide that contains at least one ISS.

The invention also provides for a method of screening for human immunostimlatory activity of oligonucleotides comprising the steps of: (a) providing macrophage cells and an aliquot of the oligonucleotide to be tested; (b) incubating the cells and oligonucleotide of step a) for an appropriate length of time; and (c) determining the relative amount of Th1-biased cytokines in the cell culture supernatant.

The invention also provides for a methods of treating individuals in need of immune modulation comprising administration of a composition comprising an immunomodulatory oligonucleotide that contains at least one ISS, including, but not limited to, individuals suffering from cancer, allergic diseases and infectious diseases. Further embodiments provide methods from treating individuals infected with hepatitis B virus, papillomavirus, and human immunodeficiency virus.

In another embodiment, the invention provides methods of preventing an infectious disease in an individual comprising administration of an immunomodulatory composition comprising and ISS and antigen.

Further embodiments include methods of preventing infectious disease due to viral infection, including, but not limited to, those diseases due to infection by hepatitis B virus, influenza virus, herpes virus, human immunodeficeincy virus and papillomavirus.

Further embodiments include methods of preventing infectious disease due to bacterial infection, including, but not limited to, those diseases due to infection by *Hemophilus influenza*, *Mycobacterium tuberculosis* and *Bordetella pertussis*.

Further embodiments include methods of preventing infectious disease due to parasitic infection, including, but not limited to, those diseases due to infection by malarial plasmodia, Leishmania species, Trypanosoma species and Schistosoma species.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the level of IFN-γ found in the culture supernatant of splenocytes after exposure to oligonucleotides for 48 hours. See Table 1 for identification of oligonucleotides.

Figure 2 is a graph depicting the level of IL-12 found in the culture supernatant of splenocytes after exposure to oligonucleotides for 48 hours. See Table 1 for identification of oligonucleotides.

Figure 3 is a graph depicting the level of IL-6 found in the culture supernatant of splenocytes after exposure to oligonucleotides for 48 hours. See Table 1 for identification of oligonucleotides.

Figure 4 presents a graph depicting the level of IL-6 found in the culture supernatant of splenocytes after exposure to oligonucleotides for 48 hours. See Table 2 for identification of oligonucleotides.

Figure 5 presents a graph depicting the level of IL-12 found in the culture supernatant of splenocytes after exposure to oligonucleotides for 48 hours. See Table 2 for identification of oligonucleotides.

Figure 6 presents a graph showing the efficacy of various oligonucleotides comprising modified cytosines to stimulate proliferation of splenocytes. Cell proliferation determined after 48 hours in culture. See Table 2 for identification of oligonucleotides.

Figure 7 is a graph depicting serum levels of anti-Amb al IgE generated in treated animals.

Figure 8 is a graph depicting serum levels of anti-Amb al IgG1 generated in treated animals.

Figure 9 is a graph depicting serum levels of anti-Amb al IgG2a generated in treated animals.

Figure 10 is a graph depicting CTL responses from splenocytes of treated animals.

Figure 11 is a graph depicting CTL responses from splenocytes of treated animals.

Figure 12 is a graph depicting IFN-γ produced from splenocytes of treated animals.

Figure 13 is a graph depicting IL-10 produced from splenocytes of treated animals.

Figure 14 is a graph depicting serum levels of anti-HBsAg antibodies four weeks after primary immunization.

Figure 15 is a graph depicting serum levels of anti-HBsAg antibodies one week after secondary immunization.

Figure 16 is a graph depicting serum levels of anti-HBsAg antibodies four weeks after secondary immunization.

MODES FOR CARRYING OUT THE INVENTION

It has now been found that a particular set of octanucleotide sequences within oligonucleotide sequences renders the oligonucleotide capable of modulating an immune response.

Such oligonucleotide sequences comprise an immunostimulatory octanucleotide sequence (ISS). Compositions of the invention comprise the ISS octanucleotide-containing oligonucleotide alone or in conjunction with a immunomodulatory agent, such as a peptide, an antigen and/or an additional adjuvant. The oligonucleotides themselves have been found to have adjuvant activity and are suitable for use as adjuvants alone and have also been found to potentiate the effect of another adjuvant.

Previously described immunostimulatory sequences have been defined as containing a hexamer sequence with a central CpG dinucleotide. Unfortunately, relying on the hexamer sequence to predict immunostimulatory activity yields, for the most part, immunologically inactive oligonucleotides. For instance, as shown in Example 1, 5 different oligonucleotides with the hexamer AACGTT had clearly demonstrable immunostimulatory activity whereas 5 other oligonucleotides with AACGTT had much reduced immunostimulatory activity. Thus, the previous hexamer algorithm is not predictive of immunostimulatory activity.

The ISS of the present invention comprise an octanucleotide sequence which comprises the previously described hexamer and two additional nucleotides 3' of the hexamer. Preferably, the ISS octamer comprises 5'-purine, purine, cytosine, guanine, pyrimidine, pyrimidine, cytosine, guanine-3' or the ISS octamer comprises 5'-purine, purine, cytosine, guanine, pyrimidine, pyrimidine, cytosine, cytosine-3'. More preferably, the ISS octanucleotide comprises 5'-GACGTTCG-3' or 5'-GACGTTCC-3'. Still more preferably, the ISS octanucleotide comprises 5'-AACGTTCG-3' or 5'-AACGTTCC-3'. The present invention demonstrates that, relative to the hexameric ISS sequence, the ISS octanucleotide is a reliable predictor of immunostimulatory activity in oligonucleotides.

In another embodiment, the ISS oligonucleotide of the present invention can also comprise a CG dinucleotide in which the C residue is modified by addition to C-5 and/or C-6 of an electron-withdrawing moiety ("modified ISS"). When the same cytosine is methylated, all immunostimulatory activity of the oligonucleotide is lost. Preferably, in such compositions, the cytosine in the third position from the 5' end can be substituted with a cytosine analog, preferably 5-bromocytidine, fluorinated cytosine, or chlorinated cytosine. Some of the modified ISS have approximately the same, if not greater, immunostimulatory activity relative to the same sequence without a modified base.

The ISS oligonucleotide of the present invention can comprise any other physiologically acceptable modified nucleotide base.

The invention also provides a method and compositions for a general stimulation of an immune response through the adjuvant-like effect of an administered ISS.

The invention also provides compositions for the enhancement of an immune response which comprise an ISS-antigen conjugate. An ISS-antigen conjugate can be formed through covalent and/or non-covalent interactions between the ISS and the antigen.

The invention also provides compositions which comprise an ISS-antigen admixture in which the ISS and the antigen are proximately associated at a distance effective to enhance an immune response compared to the co-administration of the ISS and antigen in solution. The invention further provides compositions which comprise an encapsulating agent that can maintain

the ISS and antigen in proximate association until the ISS-antigen complex is available to the target. In an ISS-antigen admixture, the ISS and antigen are maintained in proximate association such that both ISS and antigen can be taken up by the same target cell. Further, ISS and antigen in an admixture are maintained at concentrations effective to modulate an immune response. Preferably, the ISS and antigen are proximately associated at a distance of about 0.04 μm to about 100 μm, more preferably, at a distance of about 0.1 μm to about 20 μm, even more preferably, at a distance of about 0.15 μm to about 10 μm. Targets of the ISS-antigen conjugate or the ISS-antigen admixture include, but are not limited to, antigen presenting cells (APCs), such as macrophages, dendritic cells, and/or lymphocytes, lymphatic structures, such as lymph nodes and/or the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found, such as skin, lungs, and/or gastrointestinal tract.

Enhancement of an immune repsonse by a composition in which an ISS and an immunomodulatory agent are proximately associated refers to a modulation of an immune response following administration of said composition as compared to the immune response following administration of the ISS and immunomodulatory agent freely soluble with respect to each other. Enhancement of an immune response includes modulation of an immune response including, but not limited to, stimulation, suppression and a shift in the type of immune response, for instance, between a Th1-type response and a Th2-type response.

The invention also provides for compositions which comprise an ISS-antigen conjugate or an ISS-antigen admixture and an adjuvant where, upon co-administration, the association of ISS-antigen and adjuvant is effective to enhance an immune response compared to the co-administration of the ISS-antigen without adjuvant. In such compositions, the adjuvant is maintained in association with ISS-antigen so as to recruit and activate target cells to the ISS-antigen.

The present invention also provides methods for the use of ISS in conjunction with an antigen in stimulation of an immune response. Preferably, as used in such methods, the ISS provides an adjuvant-like activity in the generation of a Th1-type immune response to the antigen.

Preferably, the immune response stimulated according to the invention is biased toward the Th1-type phenotype and away from the Th2-type phenotype. With reference to the invention, stimulating a Th1-type immune response can be determined *in vitro* or *ex vivo* by measuring cytokine production from cells treated with ISS as compared to those treated without ISS. Methods to determine the cytokine production of cells include those methods described herein and any known in the art. The type of cytokines produced in response to ISS treatment indicate a Th1-type or a Th2-type biased immune response by the cells. As used herein, the term "Th1-type biased" cytokine production refers to the measurable increased production of cytokines associated with a Th1-type immune response in the presence of a stimulator as compared to production of such cytokines in the absence of stimulation. Examples of such Th1-type biased cytokines include, but are not limited to, IL-2, IL-12, and IFN-γ. In contrast, "Th2-type biased cytokines" refers to those associated with a Th2-type immune response, and include, but are not limited to, IL-4, IL-5, IL-10 and IL-13. Cells useful for the determination of ISS activity include cells of the immune system,

primary cells isolated from a host and/or cell lines, preferably APCs and lymphocytes, even more preferably macrophages and T cells.

Stimulating a Th1-type immune response can also be measured in a host treated with an ISS-antigen composition and can be determined by any method known in the art including, but not limited to: (1) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS; (2) an increase in levels of IL-12, IL-18 and/or IFN (α, β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN (α, β or γ) in an ISS-antigen treated host as compared to an antigen-primed or, primed and challenged, control treated without ISS; (3) IgG2a antibody production in an ISS-antigen treated host as compared to a control treated without ISS; and/or (4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-antigen treated host as compared to an antigen-primed, or primed and challenged, control treated without ISS. A variety of these determinations can be made by measuring cytokines made by APCs and/or lymphocytes, preferably macrophages and/or T cells, *in vitro* or *ex vivo* using methods described herein or any known in the art. Methods to determine antibody production include any known in the art.

The Th1-type biased cytokine induction which occurs as a result of ISS administration produces enhanced cellular immune responses, such as those performed by NK cells, cytotoxic killer cells, Th1 helper and memory cells. These responses are particularly beneficial for use in protective or therapeutic vaccination against viruses, fungi, protozoan parasites, bacteria, allergic diseases and asthma, as well as tumors.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); and "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

Compositions comprising ISS

A composition of the subject invention is an ISS that is capable of eliciting a desired immune response. The term "ISS" as used herein refers to oligonucleotide sequences that effect a measurable immune response as measured *in vitro*, *in vivo* and/or *ex vivo*. Examples of measurable immune responses include, but are not limited to, antigen-specific antibody production,

secretion of cytokines, activation or expansion of lymphocyte populations such as NK cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, B lymphocytes, and the like. Preferably, the ISS sequences preferentially activate a Th1-type response. The oligonucleotide of the composition contains at least one octameric ISS.

The octameric ISS preferably comprises a CG containing sequence of the general octameric sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, (Cytosine or Guanine)-3'. Most preferably, the ISS comprises an octamer selected from the group consisting of: AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG.

Where the immunostimulatory oligonucleotide comprises an RNA sequence, the ISS preferably comprises a single-stranded or double-stranded sequence selected from the group consisting of AACGUUCC, AACGTTCG, GACGUUCC, and GACGUUCG.

In accordance with the present invention, the oligonucleotide contains at least one ISS, and can contain multiple ISSs. The ISSs can be adjacent within the oligonucleotide, or they can be separated by additional nucleotide bases within the oligonucleotide.

As used interchangeably herein, the terms "oligonucleotide" and "polynucleotide" include single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) and double-stranded RNA (dsRNA), modified oligonucleotides and oligonucleosides or combinations thereof. The oligonucleotide can be linearly or circularly configured, or the oligonucleotide can contain both linear and circular segments.

The ISS can be of any length greater than 6 bases or base pairs, preferably greater than 15 bases or basepairs, more preferably greater than 20 bases or base pairs in length.

In general, dsRNA exerts an immunostimulatory effect and is encompassed by the invention. Modifications of ISS include any known in the art, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group. Various such modifications are described below.

Modified Bases and Base Analogs

Oligonucleotides are polymers of nucleosides joined, generally, through phosphoester linkages. A nucleoside consists of a purine (adenine or guanine or derivative thereof) or pyrimidine (thymine, cytosine or uracil, or derivative thereof) base bonded to a sugar. The four nucleoside units (or bases) in DNA are called deoxyadenosine, deoxyguanosine, deoxythymidine, and deoxycytidine. A nucleotide is a phosphate ester of a nucleoside.

Multiple bases, sugars, or phosphates in any combination can be substituted in the ISS.

The oligonucleotide of the invention can comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides (containing deoxyribose as the principal sugar component), or, in accordance with the state of the art, modified sugars or sugar analogs can be incorporated in the ISS. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar can be in pyranosyl or in a furanosyl form. In the ISS, the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-methylribose, and

the sugar can be attached to the respective heterocyclic bases either in α or β anomeric configuration. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) *per se* is known, and need not be described here, except to the extent such preparation can pertain to any specific example.

The phosphorous derivative (or modified phosphate group) which can be attached to the sugar or sugar analog moiety in the oligonucleotides of the present invention can be a monophosphate, diphosphate, triphosphate, alkylphosphate, alkanephosphate, phosphorothioate, phosphorodithioate or the like. A phosphorothiate linkage can be used in place of a phosphodiester linkage. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, *per se*, is also known and need not be described here in detail. Peyrottes et al. (1996) *Nucleic Acids Res.* 24:1841-1848; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24:2318-2323; and Schultz et al. (1996) *Nucleic Acids Res.* 24:2966-2973. Preferably, oligonucleotides of the present invention comprise phosphorothioate linkages. Oligonucleotides with phosphorothioate backbones can be more immunogenic than those with phosphodiester backbones and appear to be more resistant to degradation after injection into the host. Braun et al. (1988) *J. Immunol.* 141:2084-2089; and Latimer et al. (1995) *Mol. Immunol.* 32:1057-1064.

The heterocyclic bases, or nucleic acid bases, which are incorporated in the ISS can be the naturally-occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally-occurring and synthetic modifications of said principal bases.

Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and that as long as other criteria of the present invention are satisfied, the ISS can include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. Preferably, however, the heterocyclic base in the ISS includes, but is not limited to, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrolo [2,3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the ISS via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

In one embodiment, the ISS comprises at least one modified base. As used herein, the term "modified base" is synonymous with "base analog", for example, "modified cytosine" is synonymous with "cytosine analog." Similarly, "modified" nucleosides or nucleotides are herein defined as being synonymous with nucleoside or nucleotide "analogs." In a preferred embodiment, a cytosine of the ISS is substituted with a cytosine modified by the addition to C-5 and/or C-6 on cytosine with an electron-withdrawing moiety. Preferably, the electron-withdrawing moiety is a halogen. Such modified cytosines can include, but are not limited to, azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, flourinated

cytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, halogenated cytosine, halogenated pyrimidine analogue, hydroxyurea, iodouracil, 5-nitrocytosine, uracil, and any other pyrimidine analog or modified pyrimidine.

Methods of modulating immune responses with ISS

In one embodiment, the invention provides compositions comprising ISS as the only immunologically active substance. Upon administration, such ISS induces a stimulation of the immune system.

In other embodiments, ISS can be administered in conjunction with one or more members of the group of immunomodulatory molecules comprising antigens (including, but not limited to, proteins, glycoproteins, polysaccharides, and lipids), and/or immunomodulatory facilitators such as co-stimulatory molecules (including, but not limited to, cytokines, chemokines, targeting protein ligand, trans-activating factors, peptides, and peptides comprising a modified amino acid) and adjuvants (including, but not limited to, alum, lipid emulsions, and polylactide/polyglycolide microparticles). The term "immunomodulatory" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, macrophages and the like; increased synthesis of immunostimulatory cytokines including, but not limited to, IL-1, IL-2, IL-4, IL-5, IL-6, IL-12, IFN-γ, TNF-α and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses. Examples of immunosuppressive effects include, but are not limited to, a reduction in antigen-specific antibody production such as reduced IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN-γ, which appears to block IL-4 induced class switch to IgE and IgG1, thereby reducing the levels of these antibody subclasses.

The ISS and the antigen and/or immunomodulatory facilitator can be administered together in the form of a conjugate or co-administered in an admixture sufficiently close in time so as to modulate an immune response. Preferably, the ISS and immunomodulatory molecule are administered simultaneously. The term "co-administration" as used herein refers to the administration of at least two different substances sufficiently close in time to modulate an immune response. Preferably, co-administration refers to simultaneous administration of at least two different substances.

As used herein, the term "conjugate" refers to a complex in which an ISS and an immunomodulatory molecule are linked. Such conjugate linkages include covalent and/or non-covalent linkages.

As used herein, the term "antigen" means a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins,

glycoproteins, polysaccharides, gangliosides and lipids; portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic. Haptens are included within the scope of "antigen." A hapten is a low molecular weight compound that is not immunogenic by itself but is rendered immunogenic when conjugated with an immunogenic molecule containing antigenic determinants.

As used herein, the term "adjuvant" refers to a substance which, when added to an immunogenic agent, nonspecifically enhances or potentiates an immune response to the agent in the recipient host upon exposure to the mixture.

In the stimulation of an immune response, most adjuvants have generally been found to stimulate macrophages at the site of injection. As described herein, ISS have been shown to stimulate cytokine production from macrophage cells and, as such, immunostimulatory polynucleotides function as adjuvants. Thus, in another embodiment, the invention provides compositions comprising ISS and an antigen. Antigens suitable for administration with ISS include any molecule capable of eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, lipids and polypeptides, as well as macromolecules such as complex carbohydrates, and phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic. Preferably, antigens of the present invention include peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *Hemophilus influenza* vaccines, gangliosides and glycoproteins.

As used herein, the term "peptide" includes peptides and proteins that are of sufficient length and composition to effect a biological response, e.g. antibody production or cytokine activity whether or not the peptide is a hapten. Typically, the peptides are of at least six amino acid residues in length. The term "peptide" further includes modified amino acids, such modifications including, but not limited to, phosphorylation, glycosylation, pegylation, lipidization and methylation.

In one embodiment, the invention provides compositions comprising ISS and antigenic peptides. Antigenic peptides can include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides.

Many antigenic peptides and proteins are known, and available in the art; others can be identified using conventional techniques. Protein antigens that can serve as immunomodulatory facilitators include, but are not limited to, the following examples. Isolated native or recombinant antigens can be derived from plant pollens (see, for example, Rafnar et al. (1991) *J. Biol. Chem.* 266:1229-1236; Breiteneder et al. (1989) *EMBO J.* 8:1935-1938; Elsayed et al. (1991) *Scand. J. Clin. Lab. Invest. Suppl.* 204:17-31; and Malley (1989) *J. Reprod. Immunol.* 16:173-186), dust mite proteins (see, for example, Chua et al. (1988) *J. Exp. Med.* 167:175-182; Chua et al. (1990) *Int. Arch. Allergy Appl. Immunol.* 91:124-129; and Joost van Neerven et al. (1993) *J. Immunol.* 151:2326-2335), animal dander (see, for example, Rogers et al. (1993) *Mol. Immunol.* 30:559-568), animal saliva, bee venom, and fungal spores. Live, attenuated and inactivated microorganisms such as HIV-1, HIV-2, herpes simplex virus, hepatitis A virus (Bradley et al. (1984) *J. Med. Virol.*

14:373-386), rotavirus, polio virus (Jiang et al. (1986) *J. Biol. Stand.* 14:103-109), hepatitis B virus, measles virus (James et al. (1995) *N. Engl. J. Med.* 332:1262-1266), human and bovine papilloma virus, and slow brain viruses can provide peptide antigens. For immunization against tumor formation, immunomodulatory peptides can include tumor cells (live or irradiated), tumor cell extracts, or protein subunits of tumor antigens. Vaccines for immuno-based contraception can be formed by including sperm proteins administered with ISS. Lea et al. (1996) *Biochim. Biophys. Acta* 1307:263.

The ISS and antigen can be administered as an ISS-antigen conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion. The association of the ISS and the antigen molecules in an ISS-antigen conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity interactions. Examples of non-covalent interactions that can couple an ISS and an antigen in an ISS-antigen conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

In another embodiment, ISS can be administered in conjunction with one or more immunomodulatory facilitator. Thus, the invention provides compositions comprising ISS and an immunomodulatory facilitator. As used herein, the term "immunomodulatory facilitator" refers to molecules which support and/or enhance the immunomodulatory activity of an ISS. Examples of immunomodulatory facilitators can include co-stimulatory molecules, such as cytokines, and/or adjuvants. The ISS and facilitator can be administered as an ISS-facilitator conjugate and/or they can be co-administered as a complex in the form of an admixture, such as in an emulsion. The association of the ISS and the facilitator molecules in an ISS-facilitator conjugate can be through covalent interactions and/or through non-covalent interactions, including high affinity and/or low affinity interactions. Examples of non-covalent interactions that can couple an ISS and a facilitator in an ISS-facilitator conjugate include, but are not limited to, ionic bonds, hydrophobic interactions, hydrogen bonds and van der Waals attractions.

Immunomodulatory facilitators include, but are not limited to, co-stimulatory molecules (such as cytokines, chemokines, targeting protein ligand, trans-activating factors, peptides, and peptides comprising a modified amino acid) and adjuvants (such as alum, lipid emulsions, and polylactide/polyglycolide microparticles).

Among suitable immunomodulatory cytokine peptides for administration with ISS are the interleukins (e.g., IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN-α, IFN-β, IFN-γ), erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF-α. Preferably, immunostimulatory peptides for use in conjunction with ISS oligonucleotides are those that stimulate Th1-type immune responses, such as IL-12 (Bliss et al. (1996) *J. Immunol.* 156:887-894), IL-18, TNF-α, β and γ, and/or transforming growth factor (TGF)-α.

Peptides administered with ISS can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments, peptide hormones such as human growth hormone, and enzymes. Immunomodulatory peptides also include peptide hormones,

peptide neurotransmitters and peptide growth factors. Co-stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptides for administration with ISS.

The invention also provides for the administration of ISS in conjunction with an adjuvant. Administration of an antigen with an ISS and an adjuvant leads to a potentiation of a immune response to the antigen and thus, can result in an enhanced immune response compared to that which results from a composition comprising the ISS and antigen alone. For example, we have shown that administration of an antigen with an ISS and an adjuvant leads to an enhanced primary immune response. Thus, in another embodiment, the invention provides compositions comprising ISS, an antigen and an adjuvant whereby the ISS/antigen/adjuvant are co-administered. Preferably, the immunogenic composition contains an amount of an adjuvant sufficient to potentiate the immune response to the immunogen. Preferably, adjuvants include, but are not limited to, oil-in-water emulsions, water-in oil elmulsions, alum (aluminum salts), liposomes and microparticles, including but not limited to, polysytrene, starch, polyphosphazene and polylactide/polyglycosides. More preferably, the ISS and antigen are co-administered with alum. More preferably, the ISS and antigen are co-administered with liposomes. Still more preferably, the ISS and antigen are co-administered with an oil-in-water emulsion.

Suitable adjuvants also include, but are not limited to, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium cell wall preparations, monophosphoryl lipid A, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875, as well as, lipid-based adjuvants and others described herein. For veterinary use and for production of antibodies in animals, mitogenic components of Freund's adjuvant (both complete and incomplete) can be used.

As with all immunogenic compositions, the immunologically effective amounts of the components must be determined empirically. Factors to be considered include the antigenicity, whether or not ISS and/or antigen will be complexed with or covalently attached to an immunomodulatory facilitator, an adjuvant or carrier protein or other carrier, route of administration and the number of immunizing doses to be administered. Such factors are known in the vaccine art and it is well within the skill of immunologists to make such determinations without undue experimentation.

The invention further provides for compositions in which ISS and an immunomodulatory molecule(s) are in proximate association at a distance effective to enhance the immune response generated compared to the administration of the ISS and the immunomodulatory molecule as an admixture. Thus, the invention provides compositions and methods of use thereof comprising an encapsulating agent that can maintain the proximate association of the ISS and immunomodulatory molecule until the complex is available to the target. Preferably, the composition comprising ISS, immunomodulatory molecule and encapsulating agent is in the form of adjuvant oil-in-water emulsions, microparticles and/or liposomes. More preferably, adjuvant oil-in-water emulsions,

microparticles and/or liposomes encapsulating an ISS-immunomodulatory molecule are in the form of particles from about 0.04 μm to about 100 μm in size, more preferably, from about 0.1 μm to about 20 μm, even more preferably, from about 0.15 μm to about 10 μm.

Colloidal dispersion systems, such as microspheres, beads, macromolecular complexes, nanocapsules and lipid-based system, such as oil-in-water emulsions, micelles, mixed micelles and liposomes can provide effective encapsulation of ISS-containing compositions.

The encapsulation composition further comprises any of a wide variety of components. These include, but are not limited to, alum, lipids, phospholipids, lipid membrane structures (LMS), polyethylene glycol (PEG) and other polymers, such as polypeptides, glycopeptides, and polysaccharides.

Polypeptides suitable for encapsulation components include any known in the art and include, but are not limited to, fatty acid binding proteins. Modified polypeptides contain any of a variety of modifications, including, but not limited to glycosylation, phosphorylation, myristylation, sulfation and hydroxylation. As used herein, a suitable polypeptide is one that will protect an ISS-containing composition to preserve the immunomodulatory activity therof. Examples of binding proteins include, but are not limited to, albumins such as bovine serum albumin (BSA) and pea albumin.

Other suitable polymers can be any known in the art of pharmaceuticals and include, but are not limited to, naturally-occurring polymers such as dextrans, hydroxyethyl starch, and polysaccharides, and synthetic polymers. Examples of naturally occurring polymers include proteins, glycopeptides, polysaccharides, dextran and lipids. The additional polymer can be a synthetic polymer. Examples of synthetic polymers which are suitable for use in the present invention include, but are not limited to, polyalkyl glycols (PAG) such as PEG, polyoxyethylated polyols (POP), such as polyoxyethylated glycerol (POG), polytrimethylene glycol (PTG) polypropylene glycol (PPG), polyhydroxyethyl methacrylate, polyvinyl alcohol (PVA), polyacrylic acid, polyethyloxazoline, polyacrylamide, polyvinylpyrrolidone (PVP), polyamino acids, polyurethane and polyphosphazene. The synthetic polymers can also be linear or branched, substituted or unsubstituted, homopolymeric, co-polymers, or block co-polymers of two or more different synthetic monomers.

PEGs constitute a diverse group of molecules. A general formula for PEGs is as follows:

$$R_1O\text{–}(CH_2CH_2O)_n\text{–}R_3$$

where R_1 and R_3 are independently H, H_3C, OH, or a linear or branched, substituted or unsubstituted alkyl group and n is an integer between 1 and about 1,000. The term "PEG" includes both unsubstituted (R_1 and R_3 = H) as well as substituted PEG. The PEGs for use in encapsulation compositions of the present invention are either purchased from chemical suppliers or synthesized using techniques known to those of skill in the art.

The term "LMS", as used herein, means lamellar lipid particles wherein polar head groups of a polar lipid are arranged to face an aqueous phase of an interface to form membrane structures.

Examples of the LMSs include liposomes, micelles, cochleates (i.e., generally cylindrical liposomes), microemulsions, unilamellar vesicles, multilamellar vesicles, and the like.

A preferred colloidal dispersion system of this invention is a liposome. In mice immunized with a liposome-encapsulated antigen, liposomes appeared to enhance a Th1-type immune response to the antigen. Aramaki et al. (1995) *Vaccine* 13:1809-1814. As used herein, a "liposome" or "lipid vesicle" is a small vesicle bounded by at least one and possibly more than one bilayer lipid membrane. Liposomes are made artificially from phospholipids, glycolipids, lipids, steroids such as cholesterol, related molecules, or a combination thereof by any technique known in the art, including but not limited to sonication, extrusion, or removal of detergent from lipid-detergent complexes. A liposome can also optionally comprise additional components, such as a tissue targeting component. It is understood that a "lipid membrane" or "lipid bilayer" need not consist exclusively of lipids, but can additionally contain any suitable other components, including, but not limited to, cholesterol and other steroids, lipid-soluble chemicals, proteins of any length, and other amphipathic molecules, providing the general structure of the membrane is a sheet of two hydrophilic surfaces sandwiching a hydrophobic core. For a general discussion of membrane structure, see *The Encyclopedia of Molecular Biology* by J. Kendrew (1994). For suitable lipids see e.g., Lasic (1993) "Liposomes: from Physics to Applications" Elsevier, Amsterdam.

Preferably, a liposomal composition is chosen that allows the membrane to be formed with reproducible qualities, such as diameter, and is stable in the presence of elements expected to occur where the liposome is to be used, such as physiological buffers and circulating molecules. Preferably, the liposome is resilient to the effects of manipulation by storage, freezing, and mixing with pharmaceutical excipients.

Lipids suitable for incorporation into lipid membrane structures include, but are not limited to, natural, semi-synthetic or synthetic mono- or di-glycerophospholipids including, but not limited to, phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylglycerols (PGs), phosphatidylinositols (PIs), phosphatidic acids (PAs), phosphatidylserines (PSs), glycero- and cardiolipins. Sphingolipids such as sphingomyelin (SM) and cerebrosides can also be incorporated. While natural phospholipids occur with the phospho moiety at the *sn*-3 position and hydrophobic chains at the *sn*-1 and *sn*-2 positions, synthetic lipids can have alternative stereochemistry with, e.g., the phospho group at the *sn*-1 or *sn*-2 positions. Furthermore, the hydrophobic chains can be attached to the glycerol backbone by acyl, ether, alkyl or other linkages. Derivatives of these lipids are also suitable for incorporation into liposomes. Derivatives suitable for use include, but are not limited to, haloalkyl derivatives, including those in which all or some of the hydrogen atoms of the alkyl chains are substituted with, e.g., fluorine. In addition, cholesterol and other amphipathic steroids, bolaamphiphiles (lipids with polar moieties at either end of the molecule which form monolayer membranes) and polyglycerolmonoalkylthers can also be incorporated. Liposomes can be composed of a single lipid or mixtures of two or more different lipids.

In one embodiment, the lipid bilayer of the liposome is formed primarily from phospholipids. Preferably, the phospholipid composition is a complex mixture, comprising a combination of PS and additional lipids such as PC, PA, PE, PG and SM, PI, and/or cardiolipin (diphosphatidylglycerol). If

desired, SM can be replaced with a greater proportion of PC, PE, or a combination thereof. PS can be optionally replaced with PG. The composition is chosen so as to confer upon the LMS both stability during storage and administration.

Practitioners of ordinary skill will readily appreciate that each phospholipid in the foregoing list can vary in its structure depending on the fatty acid moieties that are esterified to the glycerol moiety of the phospholipid. Generally, most commercially available forms of a particular phospholipid can be used. However, phospholipids containing particular fatty acid moieties may be preferred for certain applications.

A general process for preparing liposomes containing ISS-containing compositions is as follows. An aqueous dispersion of liposomes is prepared from membrane components, such as phospholipids (e.g. PS, PC, PG, SM and PE) and glycolipids according to any known methods. See, e.g., *Ann. Rev. Biophys. Bioeng.* 9:467 (1980). The liposomes can further contain sterols, dialkylphosphates, diacylphosphatidic acids, stearylamine, α-tocopherol, etc., in the liposomal membrane.

To the liposomal dispersion thus prepared is added an aqueous solution of the ISS-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at a temperature above the phase transition temperature of the membrane or above 40°C, followed by cooling to thereby prepare liposomes containing the ISS-containing composition in the liposomal membrane.

Alternatively, the desired liposomes can also be prepared by previously mixing the above-described membrane components and ISS-containing composition and treating the mixture in accordance with known methods for preparing liposomes.

The lipid vesicles can be prepared by any suitable technique known in the art. Methods include, but are not limited to, microencapsulation, microfluidization, LLC method, ethanol injection, freon injection, the "bubble" method, detergent dialysis, hydration, sonication, and reverse-phase evaporation. Reviewed in Watwe et al. (1995) *Curr. Sci.* 68:715-724. For example, ultrasonication and dialysis methods generally produce small unilamellar vesicles; extrusion and reverse-phase evaporation generally produce larger sized vesicles. Techniques may be combined in order to provide vesicles with the most desirable attributes.

Optionally, the LMS also includes steroids to improve the rigidity of the membrane. Any amount of a steroid can be used. Suitable steroids include, but are not limited to, cholesterol and cholestanol. Other molecules that can be used to increase the rigidity of the membrane include, but are not limited to, cross-linked phospholipids.

Other preferred LMSs for use *in vivo* are those with an enhanced ability to evade the reticuloendothelial system, which normally phagocytoses and destroys non-native materials, thereby giving the liposomes a longer period in which to reach the target cell. Effective lipid compositions in this regard are those with a large proportion of SM and cholesterol, or SM and PI. LMSs with prolonged circulation time also include those that comprise the monosialoganglioside GM1, glucuronide, or PEG.

The invention encompasses LMSs containing tissue or cellular targeting components. Such targeting components are components of a LMS that enhance its accumulation at certain tissue or cellular sites in preference to other tissue or cellular sites when administered to an intact animal, organ, or cell culture. A targeting component is generally accessible from outside the liposome, and is therefore preferably either bound to the outer surface or inserted into the outer lipid bilayer. A targeting component can be *inter alia* a peptide, a region of a larger peptide, an antibody specific for a cell surface molecule or marker, or antigen binding fragment thereof, a nucleic acid, a carbohydrate, a region of a complex carbohydrate, a special lipid, or a small molecule such as a drug, hormone, or hapten, attached to any of the aforementioned molecules. Antibodies with specificity toward cell type-specific cell surface markers are known in the art and are readily prepared by methods known in the art.

The LMSs can be targeted to any cell type toward which a therapeutic treatment is to be directed, e.g., a cell type which can modulate and/or participate in an immune response. Such target cells and organs include, but are not limited to, APCs, such as macrophages, dendritic cells and lymphocytes, lymphatic structures, such as lymph nodes and the spleen, and nonlymphatic structures, particularly those in which dendritic cells are found.

The LMS compositions of the present invention can additionally comprise surfactants. Surfactants can be cationic, anionic, amphiphilic, or nonionic. A preferred class of surfactants are nonionic surfactants; particularly preferred are those that are water soluble. Nonionic, water soluble surfactants include polyoxyethylene derivatives of fatty alcohols, fatty acid ester of fatty alcohols and glyceryl esters, wherein the polyoxyethylene group is coupled via an ether linkage to an alcohol group. Examples include, but are not limited to, polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyexyethylene fatty acid ester and polyoxyethylene alkyl ethers.

The LMS compositions encompassed herein include micelles. The term "micelles" as used herein means aggregates which form from tenside molecules in aqueous solutions above a specific temperature (Krafft point) or a characteristic concentration, the critical micellization concentration (cmc). When the cmc is exceeded, the monomer concentration remains practically constant and the excess tenside molecules form micelles. Micelles are thermodynamically stable association colloids of surfactant substances in which the hydrophobic radicals of the monomers lie in the interior of the aggregates and are held together by hydrophobic interaction; the hydrophilic groups face the water and by solvation provide the solubility of the colloid. Micelles occur in various shapes (spheres, rods, discs) depending on the chemical constitution of the tenside and on the temperature, concentration or ionic strength of the solution. Reaching the cmc is manifest by abrupt changes in surface tension, osmotic pressure, electrical conductivity and viscosity.

A process for preparing micelles containing ISS-containing compositions is as follows. A micelle-forming surfactant, such as polyoxyethylene sorbitan fatty acid esters, polyoxyethylene castor oil derivatives, polyoxyethylene hardened castor oil derivatives, fatty acid sodium salts, sodium cholates, polyoxyethylene fatty acid ester, and polyoxyethylene alkyl ethers, alkyl glycosides, is added to water at a concentration above the cmc to prepare a micellar dispersion. To

the micellar dispersion is added an aqueous solution of an ISS-containing composition and the mixture is allowed to stand for a given period of time, preferably under warming at 40°C or higher, followed by cooling, to thereby prepare micelles containing ISS-containing compositions in the micellar membrane. Alternatively, the desired micelles can also be prepared by previously mixing the above-described micelle-forming substances and ISS-containing compositions and treating the mixture according to known methods for micelle formation.

ISS synthesis

a) ISS

The ISS can be synthesized using techniques and nucleic acid synthesis equipment which are well known in the art including, but not limited to, enzymatic methods, chemical methods, and the degradation of larger oligonucleotide sequences. See, for example, Ausubel et al. (1987); and Sambrook et al. (1989). When assembled enzymatically, the individual units can be ligated, for example, with a ligase such as T4 DNA or RNA ligase. U.S. Patent No. 5,124,246. Chemical synthesis of oligonucleotides can involve conventional automated methods, such as the phosphoramidite method disclosed by Warner et al. (1984) *DNA* 3:401. See also U.S. Patent No. 4,458,066. Oligonucleotide degradation can be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675.

The ISS can also be isolated using conventional polynucleotide isolation procedures. Such procedures include, but are not limited to, hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences, antibody screening of expression libraries to detect shared structural features and synthesis of particular native sequences by the polymerase chain reaction.

Circular ISS can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS is obtained through isolation or through recombinant methods, the ISS will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using any method described in the literature. See, for instance, Gao et al. (1995) *Nucleic Acids Res.* 23:2025-2029; and Wang et al. (1994) *Nucleic Acids Res.* 22:2326-2333.

The ISS can also contain phosphorous based modified oligonucleotides. These can be synthesized using standard chemical transformations. The efficient solid-support based construction of methylphosphonates has also been described. The synthesis of other phosphorous based modified oligonucleotides, such as phosphotriesters (Miller et al. (1971) *JACS* 93:6657-6665), phosphoramidates (Jager et al. (1988) *Biochem.* 27:7247-7246), and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used. Stirchak et al. (1989) *Nucleic Acids Res.* 17:6129-6141.

The techniques for making phosphate group modifications to oligonucleotides are known in the art. For review of one such useful technique, an intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phosphorothioates. The same general

technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. See also, U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103; and 5,453,496.

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also been described (including, but not limited to, e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

b) Immunomodulatory Molecules

Attenuated and inactivated viruses are sutiable for use herein as the antigen. Preparation of these viruses is well-known in the art. Polio virus can be inactivated by chemical agents such as beta-propiolactone. Jiang et al. (1986). The growth of attenuated strains of Hepatitis A virus has been described (Bradley et al. (1984)), as well as the growth of attenuated measles virus (James et al. (1995). Additionally, attenuated and inactivated viruses such as HIV-1, HIV-2, herpes simplex virus, hepatitis B virus, rotavirus, human and non-human papillomavirus and slow brain viruses can provide peptide antigens.

Allergens are suitable for use herein as immunomodulatory molecules. Preparation of many allergens is well-known in the art, including, but not limited to, preparation of ragweed pollen allergen Antigen E (*Amb al*) (Rafnar et al. 1991), major dust mite allergens *Der pI* and *Der PII* (Chua et al. (1988); and Chua et al. (1990)), white birch pollen *Betvl* (Breitneder et al. 1989), domestic cat allergen *Fel dl* (Rogers et al. (1993), and protein antigens from tree pollen (Elsayed et al. (1991)). Preparation of protein antigens from grass pollen for *in vivo* administration has been reported. Malley (1989).

Immunomodulatory peptides can be native or synthesized chemically or enzymatically. Any method of chemical synthesis known in the art is suitable. Solution phase peptide synthesis can be used to construct peptides of moderate size or, for the chemical construction of peptides, solid phase synthesis can be employed. Atherton et al. (1981) *Hoppe Seylers Z. Physiol. Chem.* 362:833-839. Proteolytic enzymes can also be utilized to couple amino acids to produce peptides. Kullmann (1987) *Enzymatic Peptide Synthesis*, CRC Press, Inc. Alternatively, the peptide can be obtained by using the biochemical machinery of a cell, or by isolation from a biological source. Recombinant DNA techniques can be employed for the production of peptides. Hames et al. (1987) *Transcription and Translation: A Practical Approach*, IRL Press. Peptides can also be isolated using standard techniques such as affinity chromatography.

Preferably the antigens are peptides, lipids (e.g. sterols, fatty acids, and phospholipids), polysaccharides such as those used in *H. influenza* vaccines, gangliosides and glycoproteins. These can be obtained through several methods known in the art, including isolation and synthesis

using chemical and enzymatic methods. In certain cases, such as for many sterols, fatty acids and phospholipids, the antigenic portions of the molecules are commercially available.

c) ISS-Immunomodulatory Molecule Conjugates

The ISS portion can be coupled with the immunomodulatory molecule portion of a conjugate in a variety of ways, including covalent and/or non-covalent interactions.

The link between the portions can be made at the 3' or 5' end of the ISS, or at a suitably modified base at an internal position in the ISS. If the immunomodulatory molecule is a peptide and contains a suitable reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N^4 amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS, specific labeling at one or more residues can be achieved.

Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, the immunomodulatory molecule of interest.

Where the immunomodulatory molecule is a peptide, this portion of the conjugate can be attached to the 3'-end of the ISS through solid support chemistry. For example, the ISS portion can be added to a polypeptide portion that has been pre-synthesized on a support. Haralambidis et al. (1990a) *Nucleic Acids Res.* 18:493-499; and Haralambidis et al. (1990b) *Nucleic Acids Res.* 18:501-505. Alternatively, the ISS can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS from the support, a terminal thiol group is left at the 3'-end of the oligonucleotide (Zuckermann et al. (1987) *Nucleic Acids Res.* 15:5305-5321; and Corey et al. (1987) *Science* 238:1401-1403) or a terminal amine group is left at the 3'-end of the oligonucleotide (Nelson et al. (1989) *Nucleic Acids Res.* 17:1781-1794). Conjugation of the amino-modified ISS to amino groups of the peptide can be performed as described in Benoit et al. (1987) *Neuromethods* 6:43-72. Conjugation of the thiol-modified ISS to carboxyl groups of the peptide can be performed as described in Sinah et al. (1991) *Oligonucleotide Analogues: A Practical Approach*, IRL Press. Coupling of an oligonucleotide carrying an appended maleimide to the thiol side chain of a cysteine residue of a peptide has also been described. Tung et al. (1991) *Bioconjug. Chem.* 2:464-465.

The peptide portion of the conjugate can be attached to the 5'-end of the ISS through an amine, thiol, or carboxyl group that has been incorporated into the oligonucleotide during its synthesis. Preferably, while the oligonucleotide is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl. Agrawal et al. (1986) *Nucleic Acids Res.* 14:6227-6245; Connolly (1985) *Nucleic Acids Res.* 13:4485-4502; Kremsky et al. (1987) *Nucleic Acids Res.* 15:2891-2909; Connolly (1987) *Nucleic Acids Res.* 15:3131-3139; Bischoff et al. (1987) *Anal. Biochem.* 164:336-344; Blanks et al. (1988) *Nucleic Acids Res.* 16:10283-10299; and U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802. Subsequent to deprotection, the latent

amine, thiol, and carboxyl functionalities can be used to covalently attach the oligonucleotide to a peptide. Benoit et al. (1987); and Sinah et al. (1991).

The peptide portion can be attached to a modified cytosine or uracil at any position in the ISS. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage. Ruth, *4th Annual Congress for Recombinant DNA Research*, p. 123.

An ISS-immunomodulatory molecule conjugate can also be formed through non-covalent interactions, such as ionic bonds, hydrophobic interactions, hydrogen bonds and/or van der Waals attractions.

Non-covalently linked conjugates can include a non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an ISS. Roget et al. (1989) *Nucleic Acids Res.* 17:7643-7651. Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated oligonucleotide.

Non-covalent associations can also occur through ionic interactions involving an ISS and residues within the immunomodulatory molecule, such as charged amino acids, or through the use of a linker portion comprising charged residues that can interact with both the oligonucleotide and the immunomodulatory molecule. For example, non-covalent conjugation can occur between a generally negatively-charged ISS and positively-charged amino acid residues of a peptide, e.g., polylysine and polyarginine residues.

Non-covalent conjugation between ISS and immunomodulatory molecules can occur through DNA binding motifs of molecules that interact with DNA as their natural ligands. For example, such DNA binding motifs can be found in transcription factors and anti-DNA antibodies.

The linkage of the ISS to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al. (1988) *Nucleic Acids Symp. Ser.* 19:189-192), oligonucleotide-fatty acid conjugates (Grabarek et al. (1990) *Anal. Biochem.* 185:131-135; and Staros et al. (1986) *Anal. Biochem.* 156:220-222), and oligonucleotide-sterol conjugates. Boujrad et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:5728-5731.

The linkage of the oligonucleotide to an oligosaccharide can be formed using standard known methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin. O'Shannessy et al. (1985) *J. Applied Biochem.* 7:347-355.

The linkage of a circular ISS to a peptide or antigen can be formed in several ways. Where the circular ISS is synthesized using recombinant or chemical methods, a modified nucleoside is suitable. Ruth (1991) in *Oligonucleotides and Analogues: A Practical Approach*, IRL Press. Standard linking technology can then be used to connect the circular ISS to the antigen or other peptide. Goodchild (1990) *Bioconjug. Chem.* 1:165. Where the circular ISS is isolated, or synthesized using recombinant or chemical methods, the linkage can be formed by chemically

activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or other peptide.

Additional methods for the attachment of peptides and other molecules to oligonucleotides can be found in U.S. Patent No. 5,391,723; Kessler (1992) "Nonradioactive labeling methods for nucleic acids" in Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press; and Geoghegan et al. (1992) *Bioconjug. Chem.* 3:138-146.

Assessment of immune response to ISS

Analysis (both qualitative and quantitative) of the immune response to ISS-containing compositions can be by any method known in the art, including, but not limited to, measuring antigen-specific antibody production, activation of specific populations of lymphocytes such as $CD4^+$ T cells or NK cells, and/or production of cytokines such as IFN, IL-2, IL-4, or IL-12. Methods for measuring specific antibody responses include enzyme-linked immunosorbent assay (ELISA) and are well known in the art. Measurement of numbers of specific types of lymphocytes such as $CD4^+$ T cells can be achieved, for example, with fluorescence-activated cell sorting (FACS). Cytotoxicity assays can be performed for instance as described in Raz et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:9519-9523. Serum concentrations of cytokines can be measured, for example, by ELISA. These and other assays to evaluate the immune response to an immunogen are well known in the art. See, for example, *Selected Methods in Cellular Immunology* (1980) Mishell and Shiigi, eds., W.H. Freeman and Co.

Administration of the ISS

The ISS can be administered alone or in combination with other pharmaceutical and/or immunogenic and/or immunostimulatory agents and can be combined with a physiologically acceptable carrier thereof. The effective amount and method of administration of the particular ISS formulation can vary based on the individual patient and the stage of the disease and other factors evident to one skilled in the art. The route(s) of administration useful in a particular application are apparent to one of skill in the art. Routes of administration include but are not limited to topical, dermal, transdermal, transmucosal, epidermal parenteral, gastrointestinal, and naso-pharyngeal and pulmonary, including transbronchial and transalveolar. A suitable dosage range is one that provides sufficient ISS-containing composition to attain a tissue concentration of about 1-10 μM as measured by blood levels. The absolute amount given to each patient depends on pharmacological properties such as bioavailability, clearance rate and route of administration.

As described herein, APCs and tissues with high concentration of APCs are preferred targets for the ISS-containing compositions. Thus, administration of ISS to mammalian skin and/or mucosa, where APCs are present in relatively high concentration, is preferred.

The present invention provides ISS-containing compositions suitable for topical application including, but not limited to, physiologically acceptable implants, ointments, creams, rinses and gels. Topical administration is, for instance, by a dressing or bandage having dispersed therein a delivery system, or by direct administration of a delivery system into incisions or open wounds. Creams,

rinses, gels or ointments having dispersed therein an ISS-containing composition are suitable for use as topical ointments or wound filling agents.

Preferred routes of dermal administration are those which are least invasive. Preferred among these means are transdermal transmission, epidermal administration and subcutaneous injection. Of these means, epidermal administration is preferred for the greater concentrations of APCs expected to be in intradermal tissue.

Transdermal administration is accomplished by application of a cream, rinse, gel, etc. capable of allowing the ISS-containing composition to penetrate the skin and enter the blood stream. Compositions suitable for transdermal administration include, but are not limited to, pharmaceutically acceptable suspensions, oils, creams and ointments applied directly to the skin or incorporated into a protective carrier such as a transdermal device (so-called "patch"). Examples of suitable creams, ointments etc. can be found, for instance, in the Physician's Desk Reference.

For transdermal transmission, iontophoresis is a suitable method. Iontophoretic transmission can be accomplished using commercially available patches which deliver their product continuously through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

For transdermal transmission, low-frequency ultrasonic delivery is also a suitable method. Mitragotri et al. (1995) *Science* 269:850-853. Application of low-frequency ultrasonic frequencies (about 1 MHz) allows the general controlled delivery of therapeutic compositions, including those of high molecular weight.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. Specifically, the irritation should be sufficient to attract APCs to the site of irritation.

An exemplary mechanical irritant means employs a multiplicity of very narrow diameter, short tines which can be used to irritate the skin and attract APCs to the site of irritation, to take up ISS-containing compositions transferred from the end of the tines. For example, the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France contains a device suitable for introduction of ISS-containing compositions.

The device (which is distributed in the U.S. by Connaught Laboratories, Inc. of Swiftwater, PA) consists of a plastic container having a syringe plunger at one end and a tine disk at the other. The tine disk supports a multiplicity of narrow diameter tines of a length which will just scratch the outermost layer of epidermal cells. Each of the tines in the MONO-VACC kit is coated with old tuberculin; in the present invention, each needle is coated with a pharmaceutical composition of ISS-

containing composition. Use of the device is preferably according to the manufacturer's written instructions included with the device product. Similar devices which can also be used in this embodiment are those which are currently used to perform allergy tests.

Another suitable approach to epidermal administration of ISS is by use of a chemical which irritates the outermost cells of the epidermis, thus provoking a sufficient immune response to attract APCs to the area. An example is a keratinolytic agent, such as the salicylic acid used in the commercially available topical depilatory creme sold by Noxema Corporation under the trademark NAIR. This approach can also be used to achieve epithelial administration in the mucosa. The chemical irritant can also be applied in conjunction with the mechanical irritant (as, for example, would occur if the MONO-VACC type tine were also coated with the chemical irritant). The ISS can be suspended in a carrier which also contains the chemical irritant or coadministered therewith.

Another delivery method for administering ISS-containing compositions makes use of non-lipid polymers, such as a synthetic polycationic amino polymer. Leff (1997) *Bioworld* 86:1-2.

Parenteral routes of administration include but are not limited to electrical (iontophoresis) or direct injection such as direct injection into a central venous line, intravenous, intramuscular, intraperitoneal, intradermal, or subcutaneous injection. Compositions suitable for parenteral administration include, but are not limited, to pharmaceutically acceptable sterile isotonic solutions. Such solutions include, but are not limited to, saline and phosphate buffered saline for injection of the ISS-containing compositions.

Gastrointestinal routes of administration include, but are not limited to, ingestion and rectal. The invention includes ISS-containing compositions suitable for gastrointestinal administration including, but not limited to, pharmaceutically acceptable, powders, pills or liquids for ingestion and suppositories for rectal administration.

Naso-pharyngeal and pulmonary routes of administration include, but are not limited to, by-inhalation, transbronchial and transalveolar routes. The invention includes ISS-containing compositions suitable for by-inhalation administration including, but not limited to, various types of aerosols for inhalation, as well as powder forms for delivery systems. Devices suitable for by-inhalation administration of ISS-containing compositions include, but are not limited to, atomizers and vaporizers. Atomizers and vaporizers filled with the powders are among a vairety of devices suitable for use in by-inhaltion delivery of powders. See, e.g., Lindberg (1993) Summary of Lecture at Management Forum 6-7 December 1993 "Creating the Future for Portable Inhalers."

The methods of producing suitable devices for injection, topical application, atomizers and vaporizers are known in the art and will not be described in detail.

The choice of delivery routes can be used to modulate the immune response elicited. For example, IgG titers and CTL activities were identical when an influenza virus vector was administered via intramuscular or epidermal (gene gun) routes; however, the muscular inoculation yielded primarily IgG2A, while the epidermal route yielded mostly IgG1. Pertmer et al. (1996) *J. Virol.* 70:6119-6125. Thus, one of skill in the art can take advantage of slight differences in immunogenicity elicited by different routes of administering the immunomodulatory oligonucleotides of the present invention.

The above-mentioned compositions and methods of administration are meant to describe but not limit the methods of administering the ISS-containing compositions of the invention. The methods of producing the various compositions and devices are within the ability of one skilled in the art and are not described in detail here.

Screening for ISS

The present invention also provides a method to screen for the immunomodulatory activity of ISS. In particular, the method provided allows *in vitro* screening of ISS for the ability to stimulate a Th1-type immune response *in vivo*. As described in Example 6, the screening method can involve the use of either a murine cell line, e.g., P388D.1, or a human cell line, e.g., 90196.B. Treatment of these cell lines with oligonucleotides with potential ISS activity and subsequent determination of cytokine production from the treated cells provided a reliable indication as to immunostimulatory activity of the oligonucleotide when administered *in vivo*. The use of cell lines, such as P388D.1 and/or 90109.B, allows for a readily available, consistent cell population on which the effect of the oligonucleotide composition can be measured. In general, oligonucleotides administered at concentrations ranging from 0.1 to 10 μg/ml that stimulated a production of cytokine, for example, IL-6 and/or IL-12, to a concentration > 2 ng/ml in the culture supernatant after 48 to 72 hours indicate immunomodulatory activity. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

The following examples are provided to illustrate, but not limit, the invention.

EXAMPLES

EXAMPLE 1

Stimulation of cytokine production by oligonucleotides comprising an ISS octanucleotide

As described above, ISS activity in polynucleotides was initially associated with DNA containing unmethylated CpG dinucleotides. The ISS element was further defined as a hexameric sequence, preferably the sequence 5'-Purine, Purine, C, G, Pyrimidine, Pyrimidine-3' (Krieg et al. (1995)). Unfortunately, relying on the hexamer sequence to predict immunostimulatory activity yields, for the most part, inactive oligonucleotides. Additional experimentation provided herein indicates, however, that nucleotides surrounding the ISS hexamer can contribute significantly to the immunostimulatory activity associated with the ISS element. In particular, specific ISS sequences have been identified that stimulate a Th1-type immune response. Experiments that have identified such ISS elements are described below.

Over 150 different oligonucleotides (see Table 1 for examples) were tested for immunostimulatory activity on mouse splenocytes and/or on human peripheral blood mononuclear cells (hPBMCs). Immunostimulation in response to oligonucleotide was assessed by measurement

of cytokine secretion into the culture media and by cell proliferation. Cytokine levels in the culture supernatant were determined by enzyme-linked immunosorbent assay (ELISA) tests.

The oligonucleotides were synthesized using standard solid phase oligonucleotide techniques. The solid phase ready analog monomers were purchased from Glen Research, Sterling, VA and included in the standard manner in a solid phase oligonucleotide synthesizer. The synthesis of the oligonucleotides were performed by TriLink BioTechnologies Inc., San Diego, CA.

Cells were isolated and prepared using standard techniques. hPBMCs were isolated from heparinized peripheral blood from healthy donors by ficoll Hypaque gradients. Spleens of BALB/c mice were harvested and the splenocytes isolated using standard teasing and treatment with ACK lysing buffer from BioWhittaker, Inc. Isolated cells were washed in RPMI 1640 media supplemented with 2% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine and resuspended at approximately 4 x 10^6 cells/ml in 10%FCS/RPMI (RPMI 1640 media with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine).

Generally, cell cultures were set up in triplicate with approximately 4 x 10^5 cells/well in a 96-well, flat microtiter plate in 100 μl 10%FCS/RPMI with the cells allowed to rest for at lest 1 hour after plating. For oligonucleotide activity assays, oligonucleotides were diluted in 10%FCS/RPMI and 100 μl of the desired oligonucleotide dilution was added to the appropriate well. In general, final oligonucleotide concentrations included 0.1 μg/ml, 1.0 μg/ml, and 10 μg/ml. Cells were then incubated for 1, 2, or 3 days.

To determine cell proliferation, 100 μl of supernatant was harvested from each well on appropriate days, pulsed with 1.0 μM tritiated thymidine and incubated overnight. Standard methods to assess tritiated thymidine incorporation were used to determine cell proliferation. Cytokine production by the cells was determined by ELISAs of culture supernatant using commercially-available antibodies to the cytokines.

Results of such experiments are graphically depicted in Figures 1-3. The oligonucleotides used included the following:

TABLE 1

SEQ ID NO:	Oligonucleotide Sequence	
1	tgaccgtg**aacgttcg**agatga	ISS (bold, underline)
2	tgactgtg**aacgttcg**agatga	ISS
3	tgactgtgaaggttagagatga	
4	tcatctcg**aacgttcc**acagtca	ISS
5	tcatctcgaacgttcacggtca	
6	tgactgtg**aacgttcc**agatga	ISS
7	tccat**aacgttcg**cct**aacgttcg**tc	2 x ISS
8	tgactgtgaacgttagcgatga	
9	tgactgtgaacgttagacgtga	
10	tgacgtgaacgttagagatga	
11	tgactcgtgaacgttagagatga	

All oligonucleotides used in these experiments contained a phosphorothioate backbone.

As shown in Fig. 1-3, the phosphorothioate oligonucleotides 1, 2 and 7 (SEQ ID NO:1, SEQ ID NO:2 and SEQ ID NO:7, respectively) are potent stimulators of secretion of IL-12, IFN-γ and IL-6 from murine splenocytes. These oligonucleotides also stimulate cytokine secretion from hPBMCs. All three of these oligonucleotides comprise the preferred octanucleotide sequence of 5'-Purine, Purine, Cytosine, Guanosine, Pyrimidine, Pyrimidine, Cytosine, Guanosine-3' (see Table 1).

Examples of additional oligonucleotides with immunostimulatory activity include oligonucleotides 4 and 6 (SEQ ID NO: 4 and SEQ ID NO:6). These immunostimulatory oligonucleotides also comprise a preferred octanucleotide sequence (see Table 1). Figures 1-3 and Table 1 also indicate that the inclusion of a hexameric ISS element, defined by Krieg et al. (1995) as 5'-Purine, Purine, C, G, Pyrimidine, Pyrimidine-3', in an oligonucleotide was not a reliable predictor of immunostimulatory activity for the oligonucleotide. See, for example, oligonucleotides 5, and 8-11.

EXAMPLE 2

Stimulation of cytokine production by ISS comprising modified bases

Several oligonucleotides comprising modified bases were tested for their immunostimulatory activity on mouse splenocytes and on hPBMCs. Immunostimulation in response to oligonucleotide was assessed by measurement of cytokine secretion into the culture media and by cell proliferation as described above. Cell cultures and oligonucleotide activity assays were set up and performed as described above.

Table 2

SEQ ID NO:	Oligonucleotide Sequence	
2	tgactgtg**aacgttcg**agatga	ISS (bold, underline)
12	tgactgtg**aabgttcc**agatga	b = 5-bromocytosine
13	tgactgtgaagcttagagatga	no ISS
14	tcactctcttccttactcttct	no ISS
15	tgactgtg**aabgttcg**agatga	b = 5-bromocytosine
16	tgactgtg**aabgttbg**agatga	b = 5-bromocytosine
17	tccat**gabgttcg**tgatcgt	b = 5-bromocytosine
18	tccat**aabgttcc**tgatgct	b = 5-bromocytosine
19	tccat**aabgttcg**tgatgct	b = 5-bromocytosine
20	tccat**aabgttcg**cct**aacgttcg**	b = 5-bromocytosine
21	tccat**aabgttcg**cct**aabgttcg**	b = 5-bromocytosine

Figures 4-6 depict cytokine production and cell proliferation results from an experiment in which mouse splenocytes were cultured oligonucleotides listed in Table 2, where b is 5-bromocytosine and an ISS octamer sequence is in bold and underlined. Oligonucleotides were used at a final concentration of 1.0 μg/ml or 10 μg/ml. Treatment of the cells with oligonucleotides containing at least one ISS resulted in the production of IL-6 and IL-12 from the cells, as well as a stimulation of cell proliferation. The oligonucleotides containing a modified ISS were, in general, as effective as or more effective than the oligonucleotide with an unmodified ISS. Oligonucleotides without an ISS were unable to stimulate IL-6 or IL-12 production or cell proliferation. All oligonucleotides used in this experiment contained a phosphorothioate backbone.

EXAMPLE 3

Potentiation of an immune response with adjuvant co-administration

The effect of adjuvant co-administration with antigen and ISS on an immune response to the antigen was examined using the adjuvant aluminum hydroxide (alum) and the oil-in-water elumsion adjuvant, MF59. Compositions comprising 1 μg AgE, also known as Amb al, a major allergic component of short ragweed, was injected intradermally into mice at week 0, 2, and 4. Antigen compositions used are listed in Table 3. Oligonucleotide 2 (SEQ ID NO:2) was used in the compositions as indicated.

Table 3

AgE	AgE-oligo 2 conjugate
AgE + oligo 2 mix (equivalent)	AgE + oligo 2 mix (50 μg oligo 2)
AgE and MF59	AgE-oligo 2 conjugate and MF59
AgE and alum (25 μg)	AgE-oligo 2 conjugate and alum (25 μg)
AgE and alum (800 μg)	

The amount of anti-AgE antibody in the serum of the mice was determined at day 0 and weeks 2, 4, and 6. Anti-AgE IgG1 and anti-AgE IgG2a antibody assays were performed by ELISA tests using the original AgE vaccine as the coated antigen on microtiter plates as described in Raz et al. (1996). Anti-AgE IgE was determined by standard radioimmunoassay techniques. Results of these experiments are depicted in Figures 7-9.

As shown in Figure 7, administration of antigen alone or in a mixture with ISS resulted in almost no anti-AgE IgG2a production, whereas administration of an antigen-ISS conjugate generated a significant level of anti-AgE IgG2a antibody. Simultaneous co-administration of an antigen-ISS conjugate and adjuvant MF59 resulted in an approximately two-fold increase in anti-AgE IgG2a antibody production relative to that obtained from the adminstration of the antigen-ISS conjugate alone. Thus, administration of antigen and ISS in proximate association, such as in the form of a conjugate, or co-administration of MF59 and antigen-ISS increased the primary Th1-type immune response generated by the antigen or by the antigen-ISS conjugate, respectively, indicating that the ISS has an independent adjuvant activity.

Anti-AgE IgG2a production as a result of co-administration of alum and antigen-ISS conjugate as compared to that of co-administration of antigen and alum also indicates an independent adjuvant activity associated with ISS (Fig. 9).

CpG containing oligonucleotides were recently shown to promote a Th1-type immune response when administered with antigen and incomplete Freund's adjuvant (IFA) as compared to the Th2-type response generated to the administration of antigen with IFA alone. Chu et al. (1997) *J. Exp. Med.* 10:1623-1631. In this study, the oligonucleotides were always administered in the presence of the presence of IFA. Although this study indicates that co-administration of CpG-containing oligonucleotides with an antigen and an adjuvant can result in a shift in the immune response from a Th2-type response to a Th1-type response, experiments were not performed to indicate any independent adjuvant activity for the oligonucleotide, as presented in the instant invention.

EXAMPLE 4

Selective Induction of a Th1-type Response in a Host after Administration of a Composition Comprising an ISS

As described herein, a Th1-type immune response is associated with the production of specific cytokines, such as IFN-γ, and results in production of CTLs.

To determine if a Th1-type immune response would be produced in mice receiving ISS oligonucleotide compositions according to the invention, mice were immunized with β-galactosidase (β-Gal) protein in various compositions, with and without co-administration of ISS oligonucleotides. The compositions used included 1 or 10 μg β-Gal and are listed in Table 4.

Table 4

β-Gal	β-Gal-oligo 2 conjugate
β-Gal-oligo 2 mix (equivalent)	β-Gal-oligo 2 mix (50 μg oligo 2)
1 μg β-Gal/Alum	

BALB/c mice were injected intradermally with the amounts and compositions shown above and sacrificed 2 weeks after injection. Their antigen dependent CTL responses and cytokine secretion profile were tested *in vitro*. CTL responses were determined as described in Sato et al. (1996). Cytokine secretion was determined by ELISA tests. Naïve mice are also included in the experiment. Results are depicted in Figures 10-13.

At an early time point in the immune response, two weeks after administration of the compositions, CTL activity was found from cells of mice receiving 10 μg antigen conjugated with an ISS (Fig. 10) Splenocytes from mice receiving 1 μg βgal conjugated with ISS generated an amount of CTL activity comparable to that of those receiving 10 μg βgal conjugated with ISS (Fig. 11). IFN-γ, a Th1-biased cytokine, was produced only from cells of mice which had received βgal conjugated with ISS (Fig. 12). Cells from these mice also produced IL-10, a Th2-biased cytokine (Fig. 13).

EXAMPLE 5

Primate immune response to antigen-ISS compositions

To examine the immunomodulatory activity of ISS beyond *in vitro* and murine experiments, immune responses in the presence of ISS are examined in primates.

Cynomolgous monkeys were immunized intramuscularly with 10 μg hepatitis B surface antigen (HBsAg) either alone or mixed with either 50 μg of oligonucleotide 2 (SEQ ID NO:2) or 500 μg of oligonucleotide 2 at week 0, 4, and 8. Antibody responses to HBsAg were measured using Abbott Laboratories AUSAB kit at week 4 (4 weeks after first injection), week 5 (5 weeks after first injection and one week after second injection) and week 8 (8 weeks after first injection and 4 weeks after second injection). The results are shown in Figures 14, 15, and 16. At each time point examined, co-administration of antigen with ISS generally resulted in a greater antibody response to the antigen. Thus, in primates, ISS provides an adjuvant-like acitvity in the generation of an immune response to the co-administered antigen.

In the experiment with cynomolgus monkeys, ISS and antigen were administered as an admixture. To determine the immunomodulatory activity of an ISS-antigen conjugate in primates, baboons are injected with compositions comprising ISS-Amb al conjugates. At appropriate intervals, antigen specific immune responses are determined as described herein. For example,

antigen-specific serum antibody levels are determined and compared to such levels in pre-immune serum.

EXAMPLE 6

Method of screening for immunostimulatory oligonucleotides

To identify oligonucleotides with potential ISS activity, cell lines are treated with the oligonucleotides to be tested and resultant cytokine production is determined, if any. Cell lines used for the screening of ISS activity are the murine cell line P388D.1 or the human cell line 90196.B, both of which are available from the American Type Culture Collection.

Cells are grown and prepared using standard techniques. Cells are harvested during growth phase and are washed in RPMI 1640 media supplemented with 2% heat-inactivated fetal calf serum (FCS), 50 μM 2-mercaptoethanol, 1% penicillin-streptomycin, and 2 mM L-glutamine and resuspended at approximately 4×10^6 cells/ml in 10%FCS/RPMI

Cell cultures are set up in triplicate with approximately 4×10^5 cells/well in a 96-well, flat microtiter plate in 100 μl 10%FCS/RPMI with the cells allowed to rest for at lest 1 hour after plating. Oligonucleotides to be tested are diluted in 10%FCS/RPMI and 100 μl of oligonucleotide dilution is added to an appropriate well. In general, final oligonucleotide concentrations include 0.1 μg/ml, 1.0 μg/ml, and 10 μg/ml. Cells are then incubated for 1, 2, or 3 days.

To determine cell proliferation, 100 μl of supernatant is harvested from each well on appropriate days, pulsed with 1.0 μM tritiated thymidine and incubated overnight. Standard methods to assess tritiated thymidine incorporation are used to determine cell proliferation.

Cytokine production by the cells is determined by ELISAs of culture supernatant using commercially-available antibodies to the cytokines. Detection of >2 ng/ml IFN-γ and/or IL-12 in the cell culture supernatant 48 or 72 hours after addition of an oligonucleotide to the cells is indicative of ISS activity in the oligonucleotide. Production of IFN-γ and/or IL-12 in particular is indicative of activity to induce a Th1-type ISS immune response.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the descriptions and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: IMMUNOSTIMULATORY OLIGONUCLEOTIDES, COMPOSITIONS THEREOF AND METHODS OF USE THEREOF

(iii) NUMBER OF SEQUENCES: 21

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(v) COMPUTER READABLE FORM:
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(C) OPERATING SYSTEM: Windows
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGACCGTGAA CGTTCGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGACTGTGAA CGTTCGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGACTGTGAA GGTTAGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCATCTCGAA CGTTCCACAG TCA 23

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCATCTCGAA CGTTCACGGT CA 22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGACTGTGAA CGTTCCAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCCATAACGT TCGCCTAACG TTCGTC 26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGACTGTGAA CGTTAGCGAT GA 22

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGACTGTGAA CGTTAGACGT GA 22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGACGTGAAC GTTAGAGATG A 21

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TGACTCGTGA ACGTTAGAGA TGA 23

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 11...0

(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TGACTGTGAA NGTTCCAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGACTGTGAA GCTTAGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TCACTCTCTT CCTTACTCTT CT 22

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 11...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGACTGTGAA BGTTCGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 11...0
(D) OTHER INFORMATION: 5-bromocytosine

(A) NAME/KEY: Modified Base
(B) LOCATION: 15...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TGACTGTGAA BGTTBGAGAT GA 22

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 8...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TCCATGABGT TCGTGATCGT 20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCCATAABGT TCCTGATGCT 20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 8...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCCATAABGT TCGTGATGCT 20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 8...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCCATAABGT TCGCCTAACG TTCG 24

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: Modified Base
(B) LOCATION: 8...0
(D) OTHER INFORMATION: 5-bromocytosine

(A) NAME/KEY: Modified Base
(B) LOCATION: 19...0
(D) OTHER INFORMATION: 5-bromocytosine

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

TCCATAABGT TCGCCTAABG TTCG 24

CLAIMS

We claim:

1. An immunomodulatory oligonucleotide comprising at least one immunostimulatory octanucleotide sequence (ISS).

2. An immunomodulatory oligonucleotide of claim 1, wherein the ISS octanucleotide comprises the sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, Cytosine-3'.

3. An immunomodulatory oligonucleotide of claim 1, wherein the ISS octanucleotide comprises the sequence 5'-Purine, Purine, Cytosine, Guanine, Pyrimidine, Pyrimidine, Cytosine, Guanine-3'.

4. An immunomodulatory oligonucleotide of claim 1, wherein the ISS octanucleotide sequence is selected from the group of sequences consisting of AACGTTCC, AACGTTCG, GACGTTCC, and GACGTTCG.

5. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:2.

6. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:4.

7. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:1.

8. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:6.

8. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:7.

10. An immunomodulatory oligonucleotide of claim 2, wherein at least one of the cytosines of the ISS octanucleotide sequence is substituted with a modified cytosine.

11. An immunomodulatory oligonucleotide of claim 10, wherein the modified cytosine comprises an addition of an electron-withdrawing group at least to C-5.

12. An immunomodulatory oligonucleotide of claim 10, wherein the modified cytosine comprises an addition of an electron-withdrawing group at least to C-6.

13. An immunomodulatory oligonucleotide of claim 10, wherein the modified cytosine is a 5'-bromocytidine.

14. An immunomodulatory oligonucleotide of claim 10, wherein the C at the third position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine.

15. An immunomodulatory oligonucleotide of claim 10, wherein the C at the third position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine and the C at the seventh position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine.

16. An immunomodulatory oligonucleotide of claim 3, wherein at least one of the cytosines of the ISS octanucleotide sequence is substituted with a modified cytosine.

17. An immunomodulatory oligonucleotide of claim 16, wherein the modified cytosine comprises an addition of an electron-withdrawing group at least to C-5.

18. An immunomodulatory oligonucleotide of claim 16, wherein the modified cytosine comprises an addition of an electron-withdrawing group at least to C-6.

19. An immunomodulatory oligonucleotide of claim 16, wherein the modified cytosine is a 5'-bromocytidine.

20. An immunomodulatory oligonucleotide of claim 16, wherein the C at the third position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine.

21. An immunomodulatory oligonucleotide of claim 16, wherein the C at the third position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine and the C at the seventh position from the 5' end of the ISS octanucleotide is substituted with a 5'-bromocytidine.

22. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:12.

23. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:15.

24. An immunomodulatory oligonucleotide comprising the sequence of SEQ ID NO:16.

25. An immunomodulatory composition comprising
an immunomodulatory oligonucleotide according to claim 1;
and further comprising an antigen.

26. An immunomodulatory composition of claim 25, wherein the antigen is selected from the group consisting of peptides, glycoproteins, polysaccharides, and lipids.

27. An immunomodulatory composition of claim 25, wherein the antigen is conjugated to the immunomodulatory oligonucleotide.

28. An immunomodulatory composition comprising

an immunomodulatory oligonucleotide according to claim 1;

and further comprising a facilitator selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligand, a trans-activating factor, a peptide, and a peptide comprising a modified amino acid.

29. An immunomodulatory composition of claim 28, wherein the facilitator is conjugated to the immunomodulatory oligonucleotide.

30. An immunomodulatory composition comprising

an immunomodulatory oligonucleotide according to claim 1;

and further comprising an antigen;

and futher comprising an adjuvant.

31. An immunomodulatory composition of claim 30, wherein the antigen is selected from the group consisting of peptides, glycoproteins, polysaccharides, and lipids.

32. An immunomodulatory composition of claim 30, wherein the antigen is conjugated to the immunomodulatory oligonucleotide.

33. An immunomodulatory composition of claim 30, wherein the immunomodulatory oligonucleotide and the antigen are encapsulated.

34. An immunomodulatory composition of claim 33, wherein the immunomodulatory oligonucleotide and the antigen are encapsulated as microparticles.

35. An immunomodulatory composition of claim 25, wherein the immunomodulatory oligonucleotide and the antigen are proximately associated at a distance effective to enhance an immune response.

36. An immunomodulatory composition of claim 25, wherein the immunomodulatory oligonucleotide and the antigen are proximately associated to co-deliver the oligonucleotide and the antigen to an immune target.

37. An immunomodulatory composition of claim 36, wherein the immune target is a lymphatic structure.

38. An immunomodulatory composition of claim 36, wherein the immune target is a antigen presenting cell.

39. An immunomodulatory composition of claim 38, wherein the antigen presenting cell is a dendritic cell.

40. An immunomodulatory composition of claim 38, wherein the antigen presenting cell is a macrophage cell.

41. An immunomodulatory composition of claim 38, wherein the antigen presenting cell is a lymphocyte.

42. An immunomodulatory composition of claim 36, wherein the immunomodulatory oligonucleotide and the antigen are associated with an adjuvant.

43. An immunomodulatory composition of claim 36, wherein the immunomodulatory oligonucleotide and the antigen are associated in microparticles.

44. An immunomodulatory composition of claim 36, wherein the immunomodulatory oligonucleotide and the antigen are associated in liposomes.

45. A method of modulating an immune response comprising co-administration of an immunomodulatory composition comprising an antigen and an oligonucleotide according to claim 1.

46. The method of claim 45, wherein the modulating of an immune response comprises induction of a Th1 response.

47. A method of modulating an immune response comprising co-administration of an immunomodulatory composition comprising an antigen conjugated to an oligonucleotide according to claim 1.

48. The method of claim 47, wherein the modulating of an immune response comprises induction of a Th1 response.

49. A method of modulating an immune response comprising the administration of an immunomodulatory composition according to claim 30.

50. The method of claim 49, wherein the modulating of an immune response comprises induction of a Th1 response.

51. A method of modulating an immune response comprising the administration of an immunomodulatory composition according to claim 28.

52. A method to screen for human immunostimulatory activity of oligonucleotides comprising the steps of:

(a) providing macrophage cells and an aliquot of an oligonucleotide to be tested;

(b) incubating the cells and oligonucleotide of step a) for an appropriate length of time;

(c) determining the relative amount of Th1-biased cytokines in the cell culture supernatant.

53. A method to screen for human immunostimlatory activity of oligonucleotides according to claim 41, wherein the cells are selected from the 90196B cell line and the P388D1 cell line.

54. A method to screen for human immunostimlatory activity of oligonucleotides according to claim 52, wherein at least one of the Th1-biased cytokines determined is interferon-gamma.

55. A method to screen for human immunostimlatory activity of oligonucleotides according to claim 52, wherein at least one of the Th1-biased cytokines determined is interleukin-12.

56. A method of treating an individual in need of immune modulation comprising administration of a composition comprising an immunomodulatory oligonucleotide of claim 1.

57. A method according to claim 56, wherein the individual is suffering from cancer.

58. A method according to claim 56, wherein the individual is suffering from an allergic disease.

59. A method according to claim 58, wherein the individual is suffering from asthma.

60. A method according to claim 56, wherein the individual is suffering from an infectious disease.

61. A method according to claim 60, wherein the individual is infected with hepatitis B virus.

62. A method according to claim 60, wherein the individual is infected with a papillomavirus.

63. A method according to claim 60, wherein the individual is infected with a human immunodeficiency virus.

64. A method of preventing an infectious disease in an individual comprising administration of an immunomodulatory composition according to claim 25.

65. A method according to claim 64, wherein the infectious disease is due to a viral infection.

66. A method according to claim 65, wherein the virus is selected from the group consisting of hepatitis B virus, influenza virus, herpes virus, human immunodeficiency virus and papillomavirus.

67. A method according to claim 64, wherein the infectious disease is due to a bacterial infection.

68. A method according to claim 67, wherein the bacteria is selected from the group consisting of Hemophilus influenza, Mycobacterium tuberculosis and Bordetella pertussis.

69. A method according to claim 64, wherein the infectious disease is due to a parasitic infection.

70. A method according to claim 75, wherein the parasitic agent is selected from a group consisting of malarial plasmodia, Leishmania species, Trypanosoma species and Schistosoma species.

Fig. 1

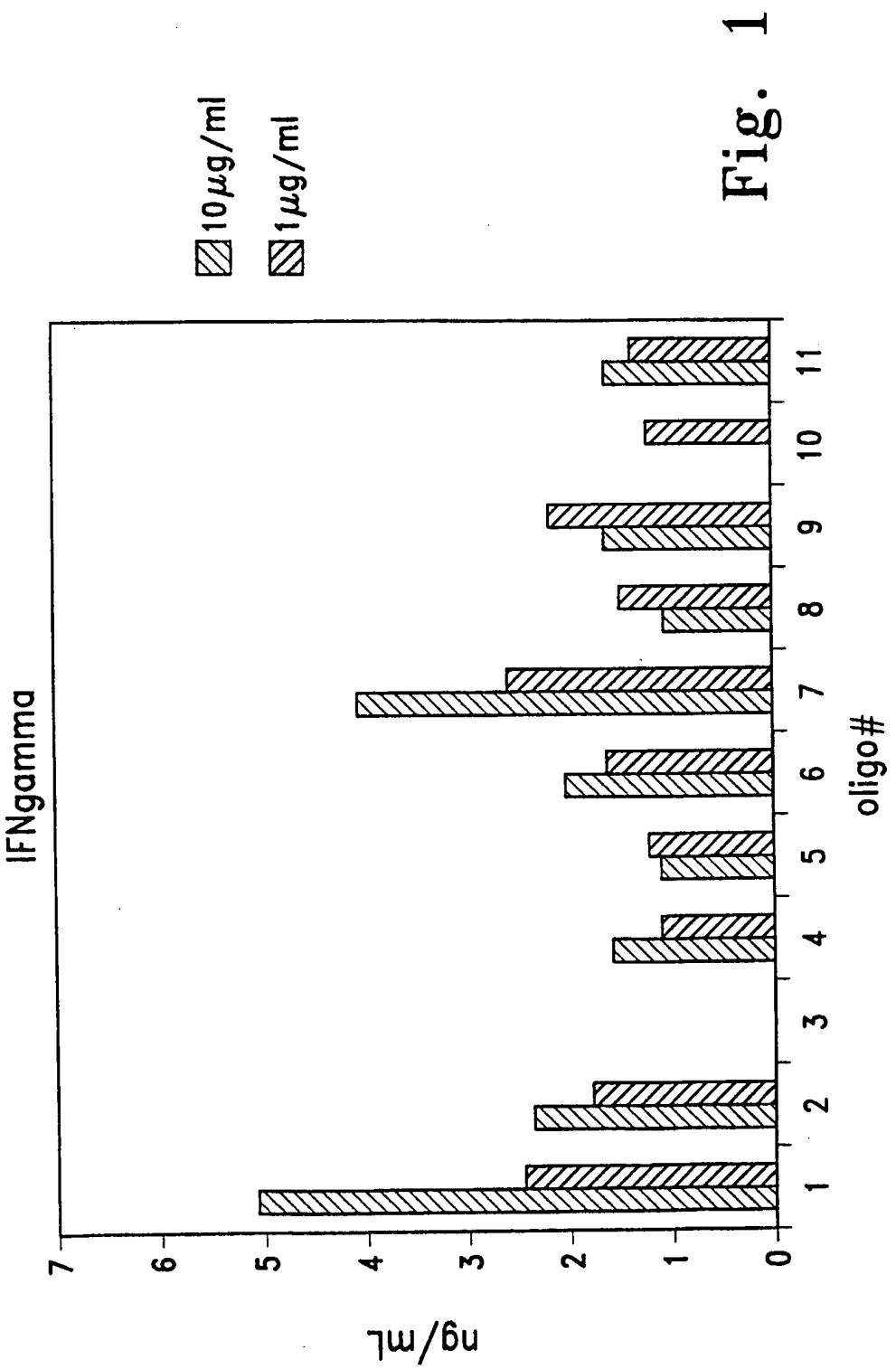


Fig. 2

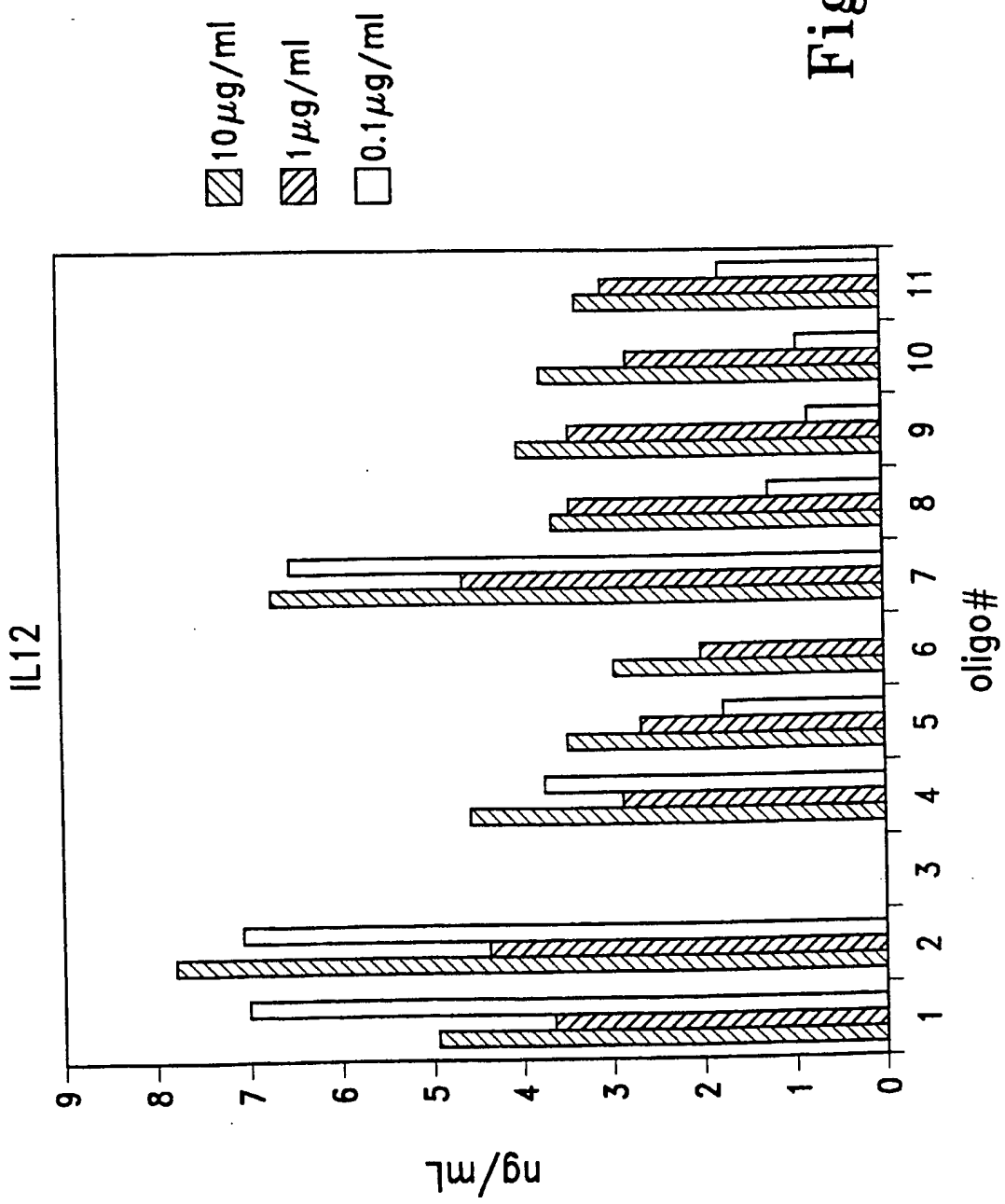
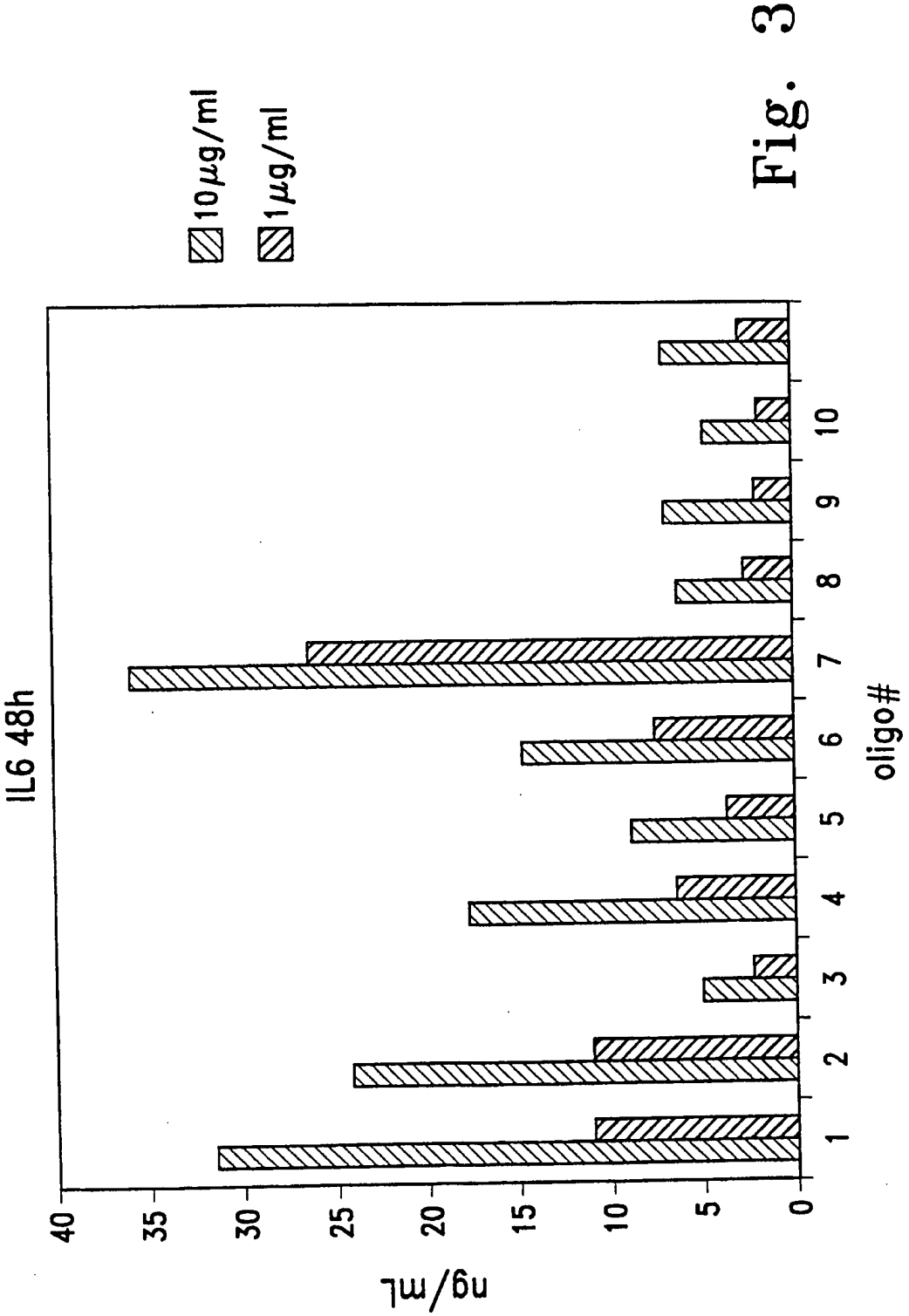

10μg/ml
1μg/ml
0.1μg/ml
IL12
ng/mL
0
1
2
3
4
5
6
7
8
9
oligo#
1
2
3
4
5
6
7
8
9
10
11

Fig. 3


10μg/ml
1μg/ml
IL6 48h
ng/mL
0
5
10
15
20
25
30
35
40
1
2
3
4
5
6
7
8
9
10
oligo#

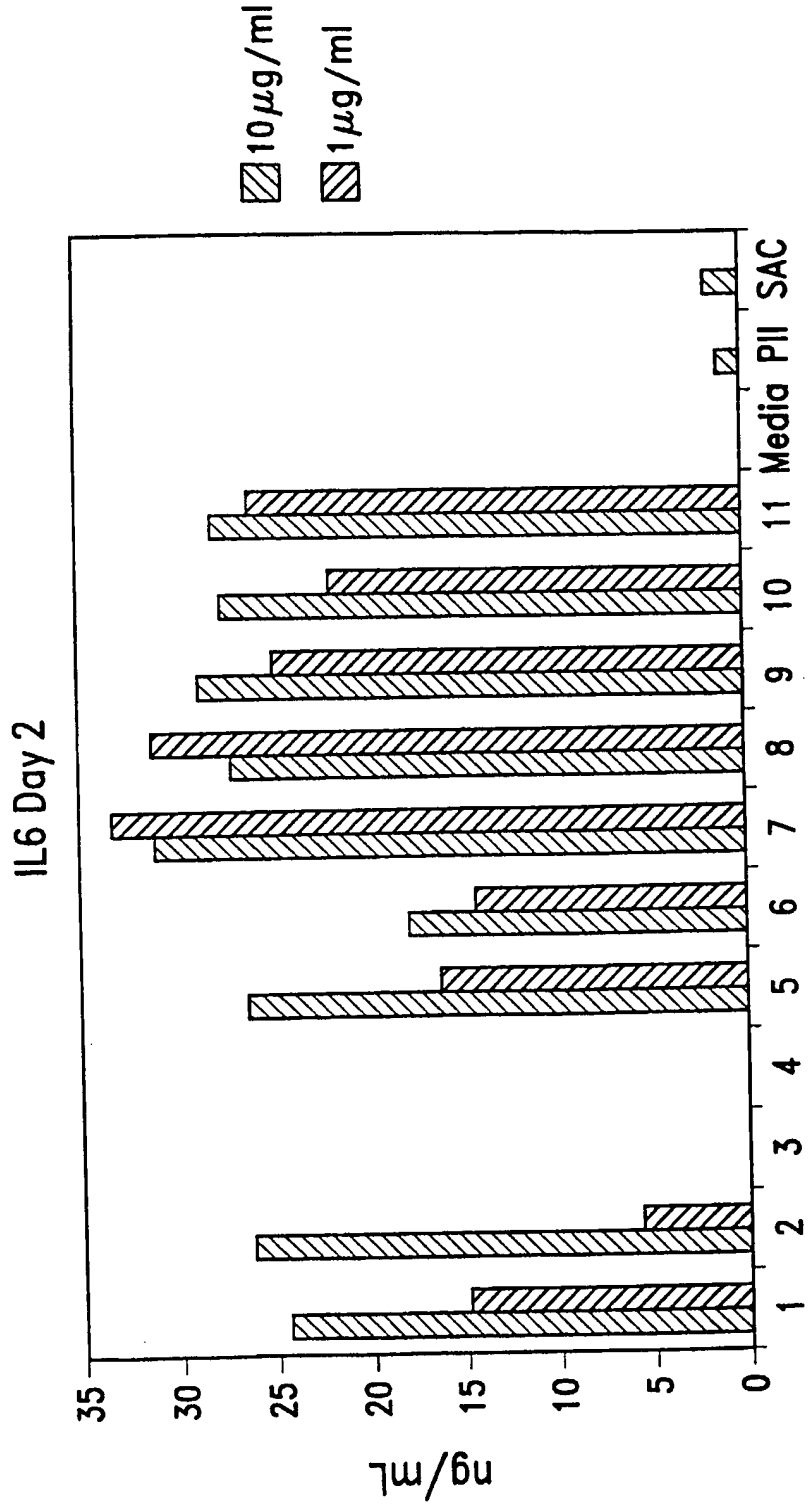

10μg/ml
1μg/ml
IL6 Day 2
ng/mL
0
5
10
15
20
25
30
35
1
2
3
4
5
6
7
8
9
10
11
Media PII SAC

Fig. 4

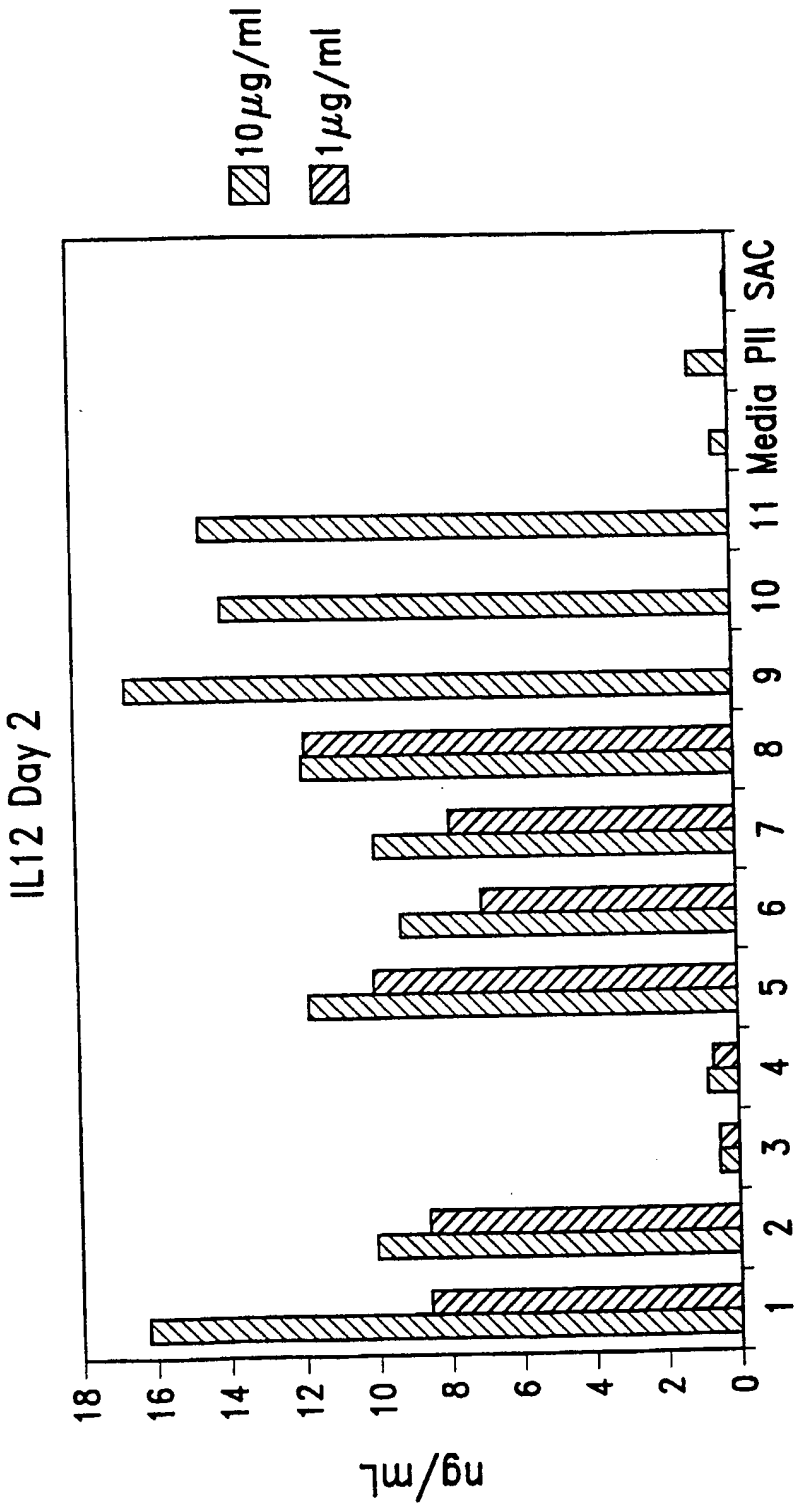

10μg/ml
1μg/ml
IL12 Day 2
ng/mL
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
7
8
9
10
11 Media PII SAC

Fig. 5

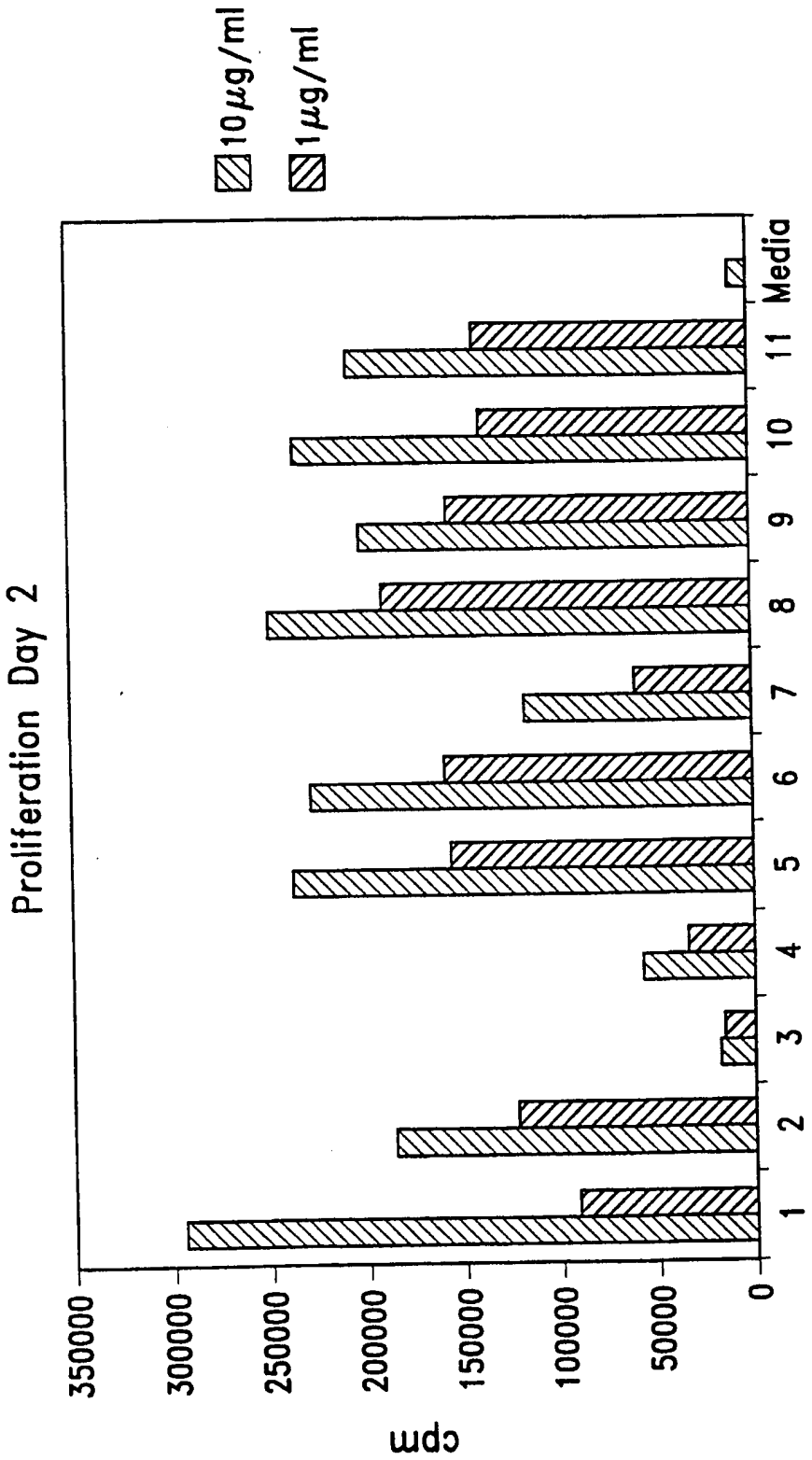

10μg/ml
1μg/ml
Proliferation Day 2
cpm
350000
300000
250000
200000
150000
100000
50000
0
1
2
3
4
5
6
7
8
9
10
11
Media

Fig. 6

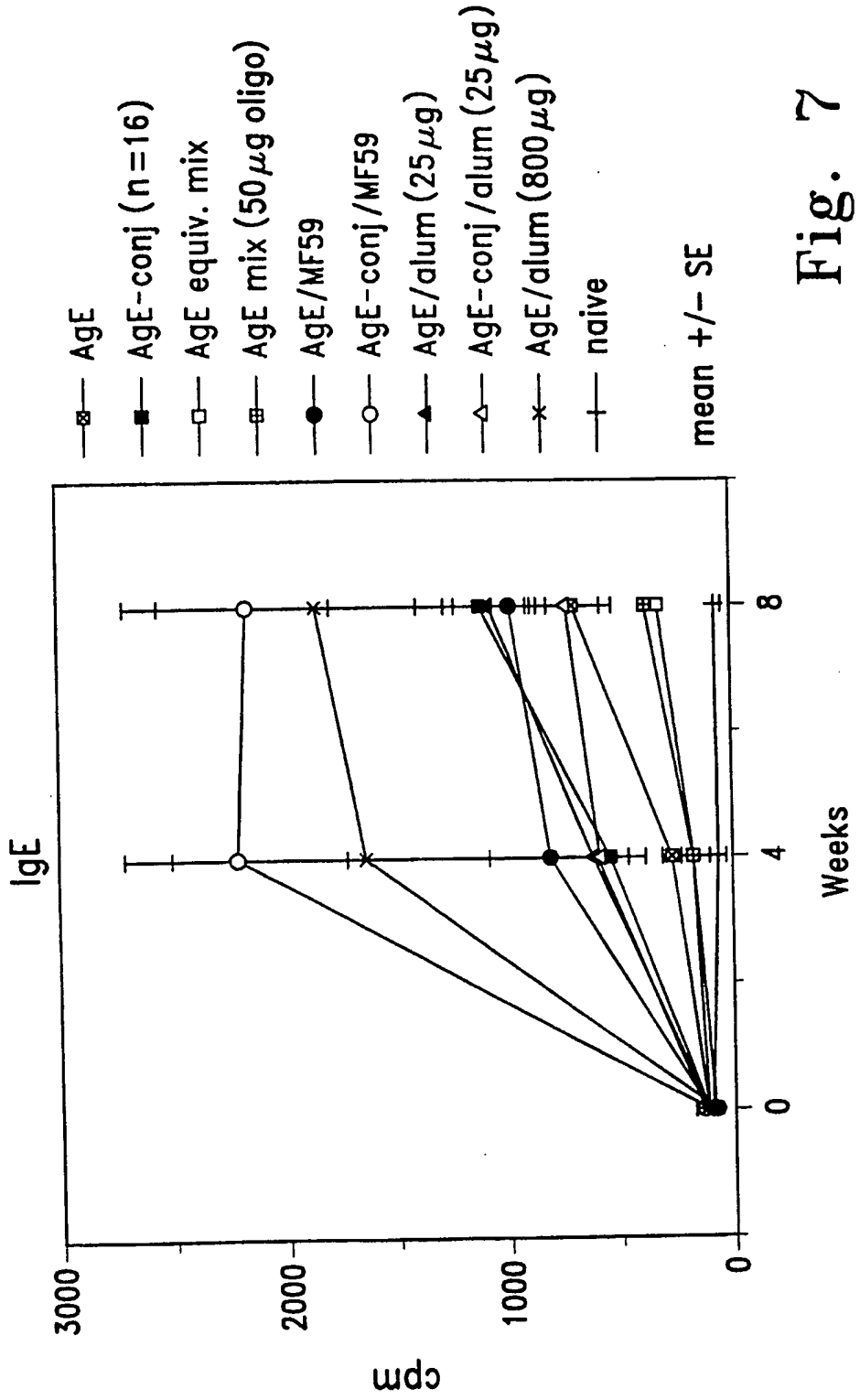

AgE
AgE-conj (n=16)
AgE equiv. mix
AgE mix (50μg oligo)
AgE/MF59
AgE-conj/MF59
AgE/alum (25μg)
AgE-conj/alum (25μg)
AgE/alum (800μg)
naive
mean +/- SE
IgE
cpm
3000
2000
1000
0
0
4
8
Weeks

Fig. 7

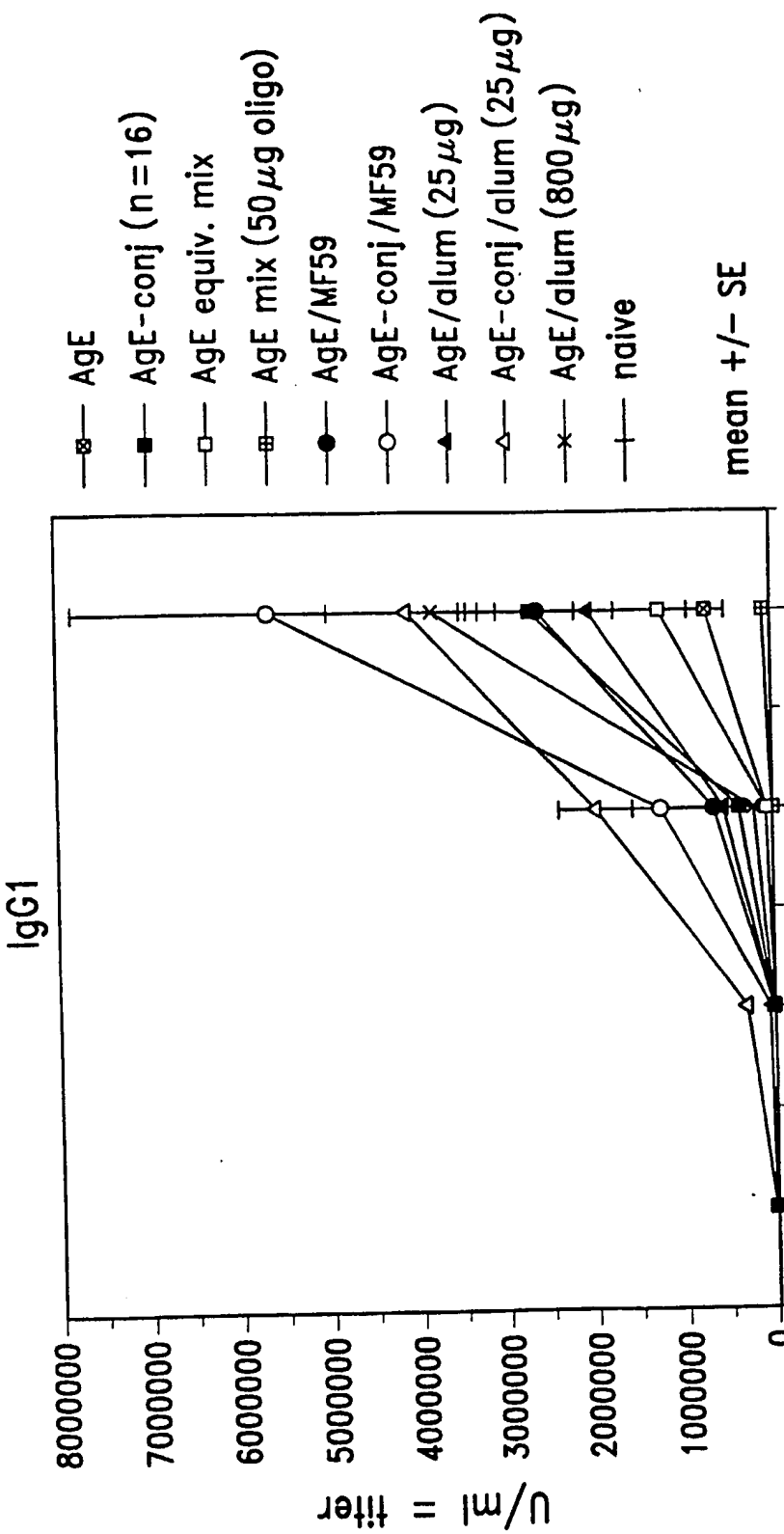

IgG1
U/ml = titer
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
0
2
4
6
Weeks
AgE
AgE-conj (n=16)
AgE equiv. mix
AgE mix (50μg oligo)
AgE/MF59
AgE-conj /MF59
AgE/alum (25μg)
AgE-conj /alum (25μg)
AgE/alum (800μg)
naive
mean +/- SE

Fig. 8

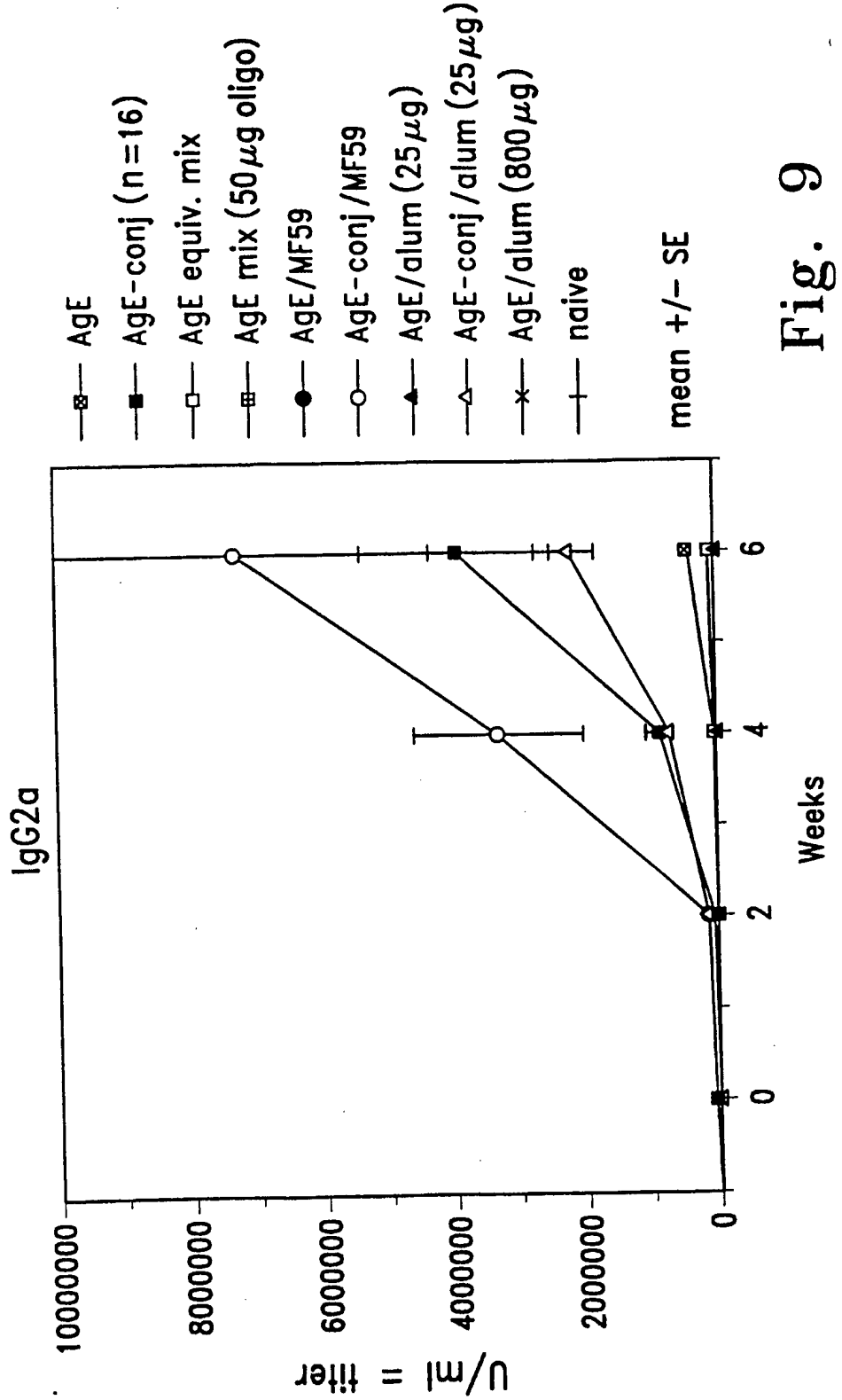

IgG2a
U/ml = titer
10000000
8000000
6000000
4000000
2000000
0
0
2
4
6
Weeks
AgE
AgE-conj (n=16)
AgE equiv. mix
AgE mix (50μg oligo)
AgE/MF59
AgE-conj/MF59
AgE/alum (25μg)
AgE-conj/alum (25μg)
AgE/alum (800μg)
naive
mean +/- SE

Fig. 9

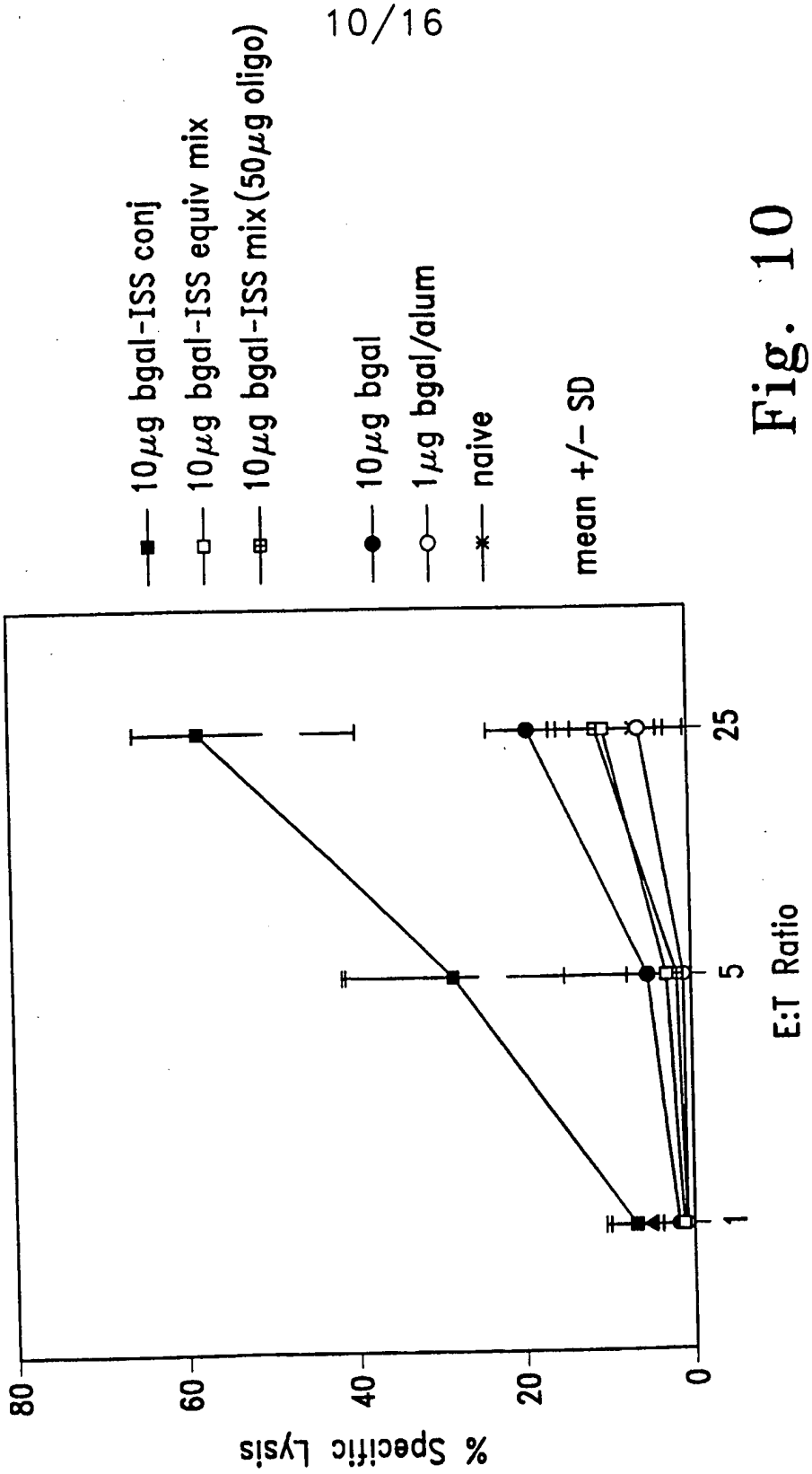

10μg bgal-ISS conj
10μg bgal-ISS equiv mix
10μg bgal-ISS mix (50μg oligo)
10μg bgal
1μg bgal/alum
naive
mean +/- SD
% Specific Lysis
0
20
40
60
80
1
5
25
E:T Ratio

Fig. 10

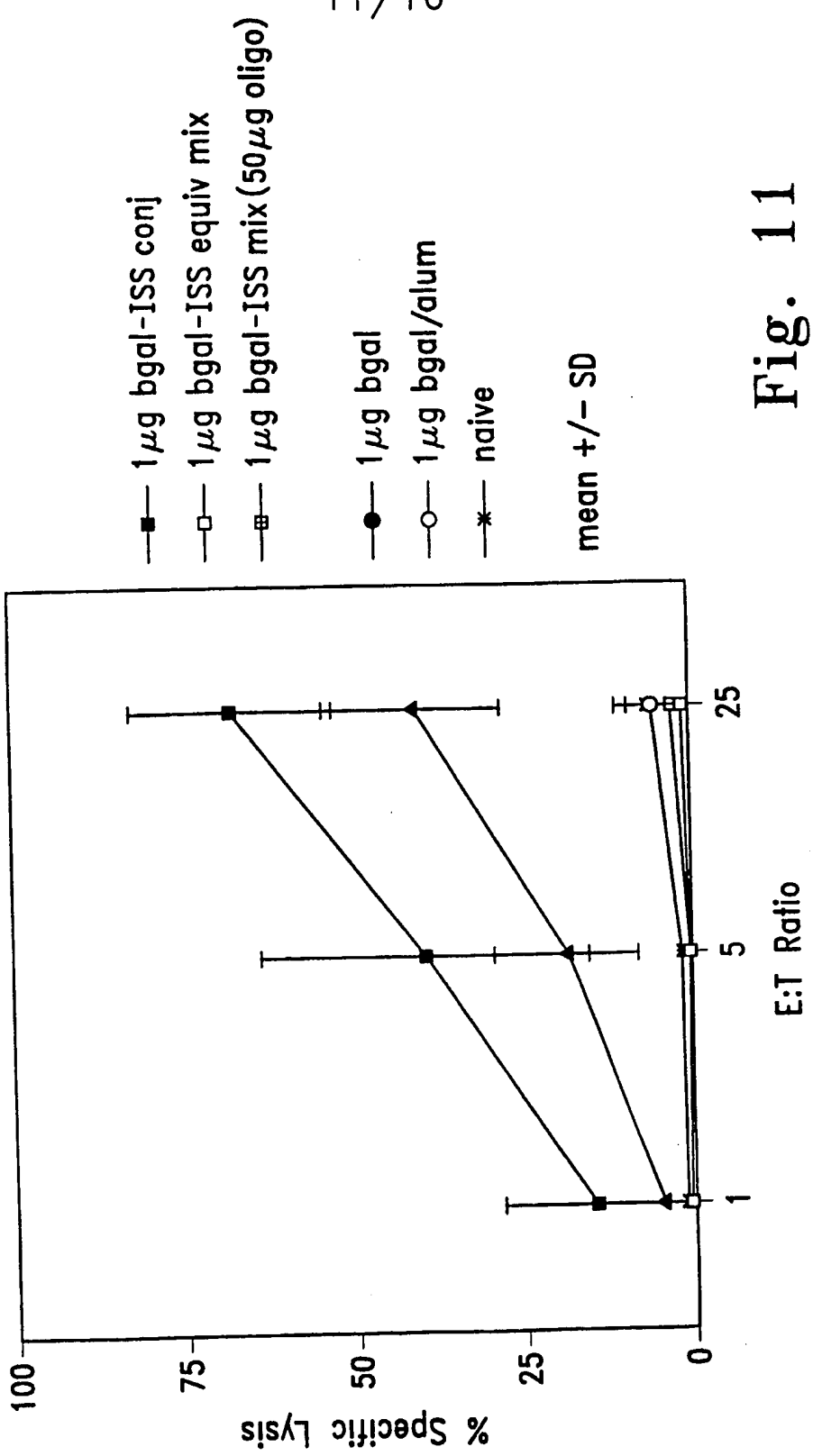
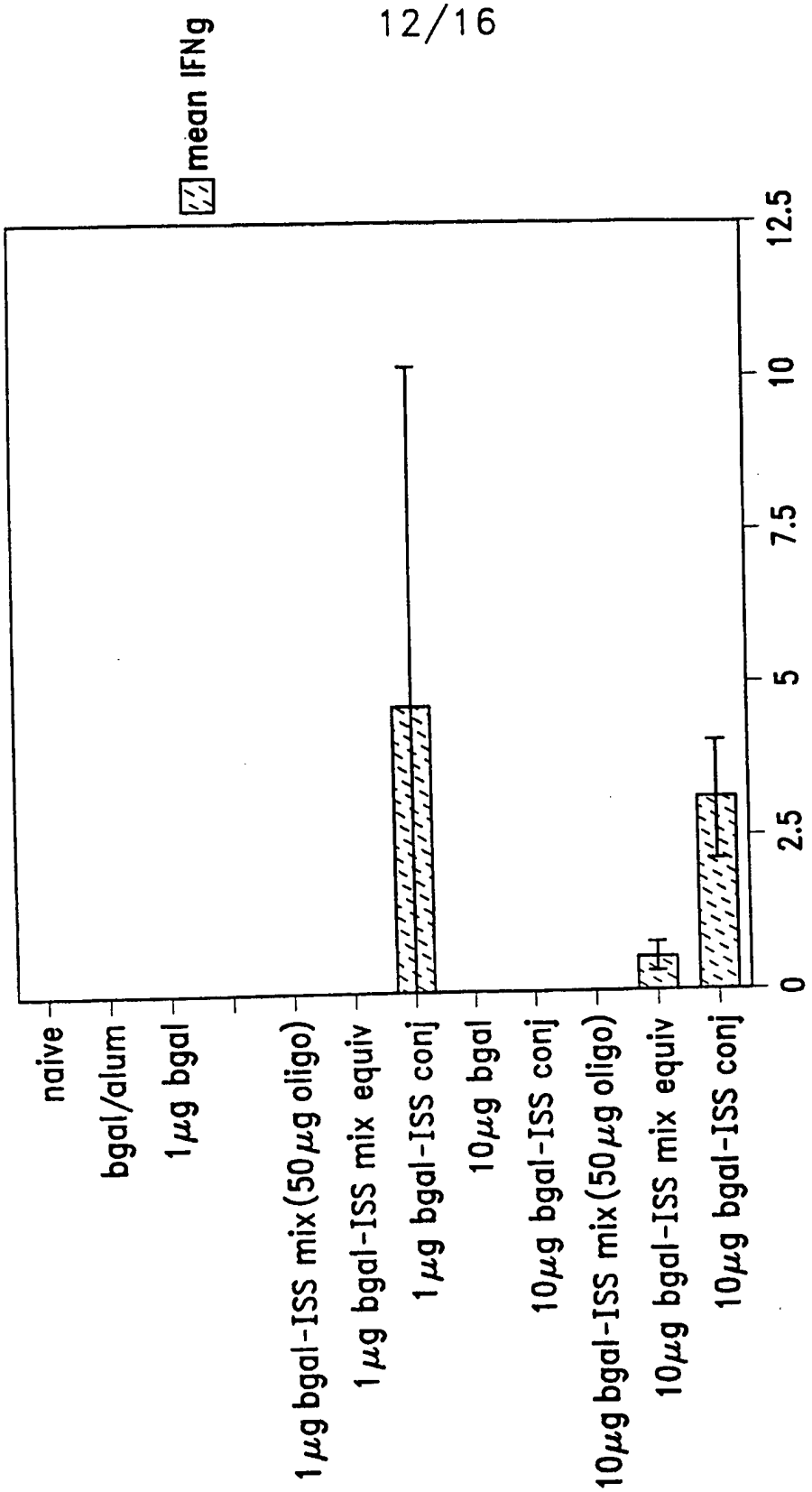

1μg bgal-ISS conj
1μg bgal-ISS equiv mix
1μg bgal-ISS mix (50μg oligo)
1μg bgal
1μg bgal/alum
naive
mean +/- SD
% Specific Lysis
100
75
50
25
0
1
5
25
E:T Ratio

Fig. 11


mean IFNg
naive
bgal/alum
1μg bgal
1μg bgal-ISS mix(50μg oligo)
1μg bgal-ISS mix equiv
1μg bgal-ISS conj
10μg bgal
10μg bgal-ISS conj
10μg bgal-ISS mix(50μg oligo)
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10μg bgal-ISS conj
0
2.5
5
7.5
10
12.5
IFNg ng/ml

Fig. 12

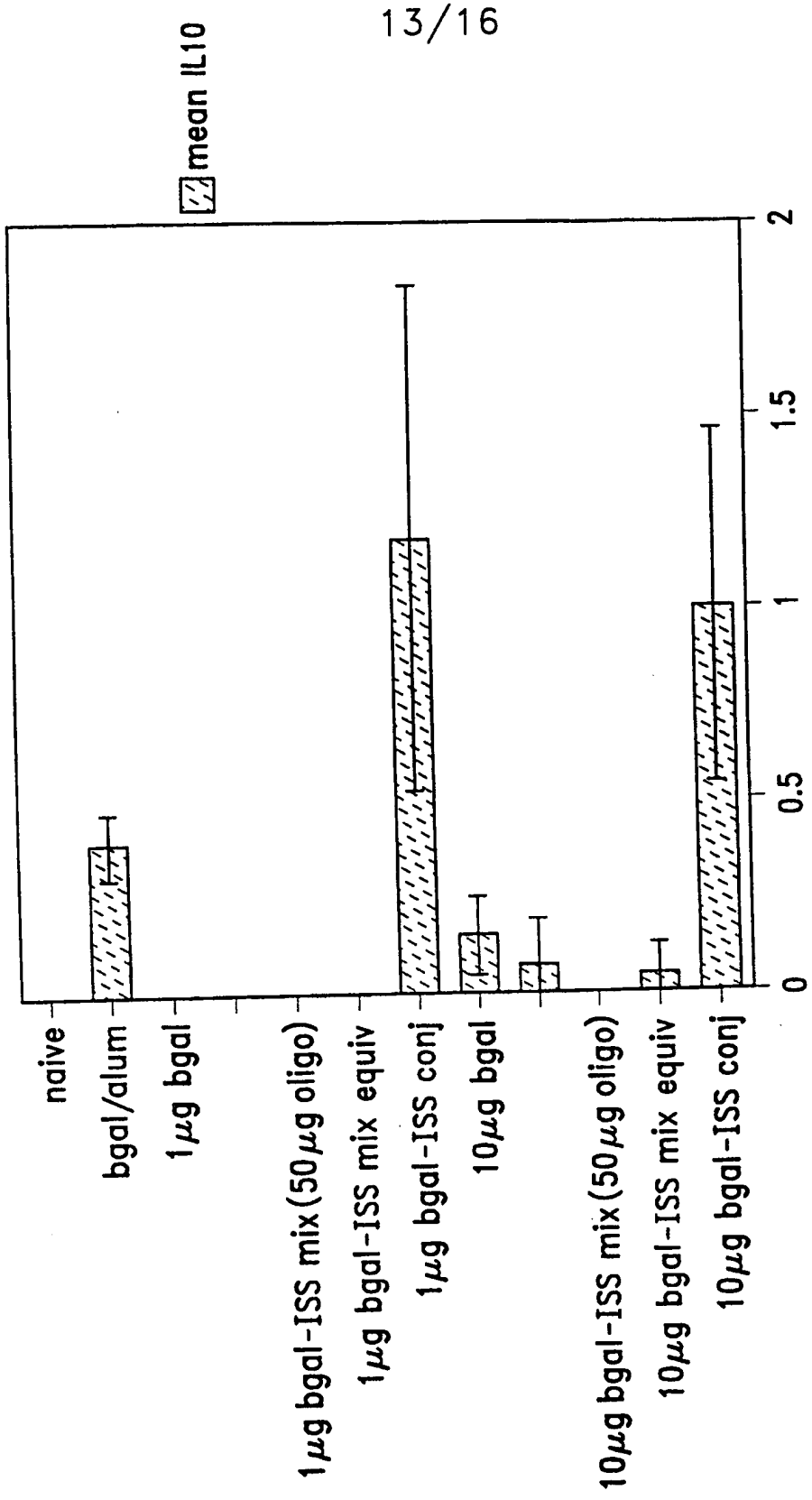
mean IL10
naive
bgal/alum
1μg bgal
1μg bgal-ISS mix (50μg oligo)
1μg bgal-ISS mix equiv
1μg bgal-ISS conj
10μg bgal
10μg bgal-ISS mix (50μg oligo)
10μg bgal-ISS mix equiv
10μg bgal-ISS conj
0
0.5
1
1.5
2
IL10 ng/ml

Fig. 13

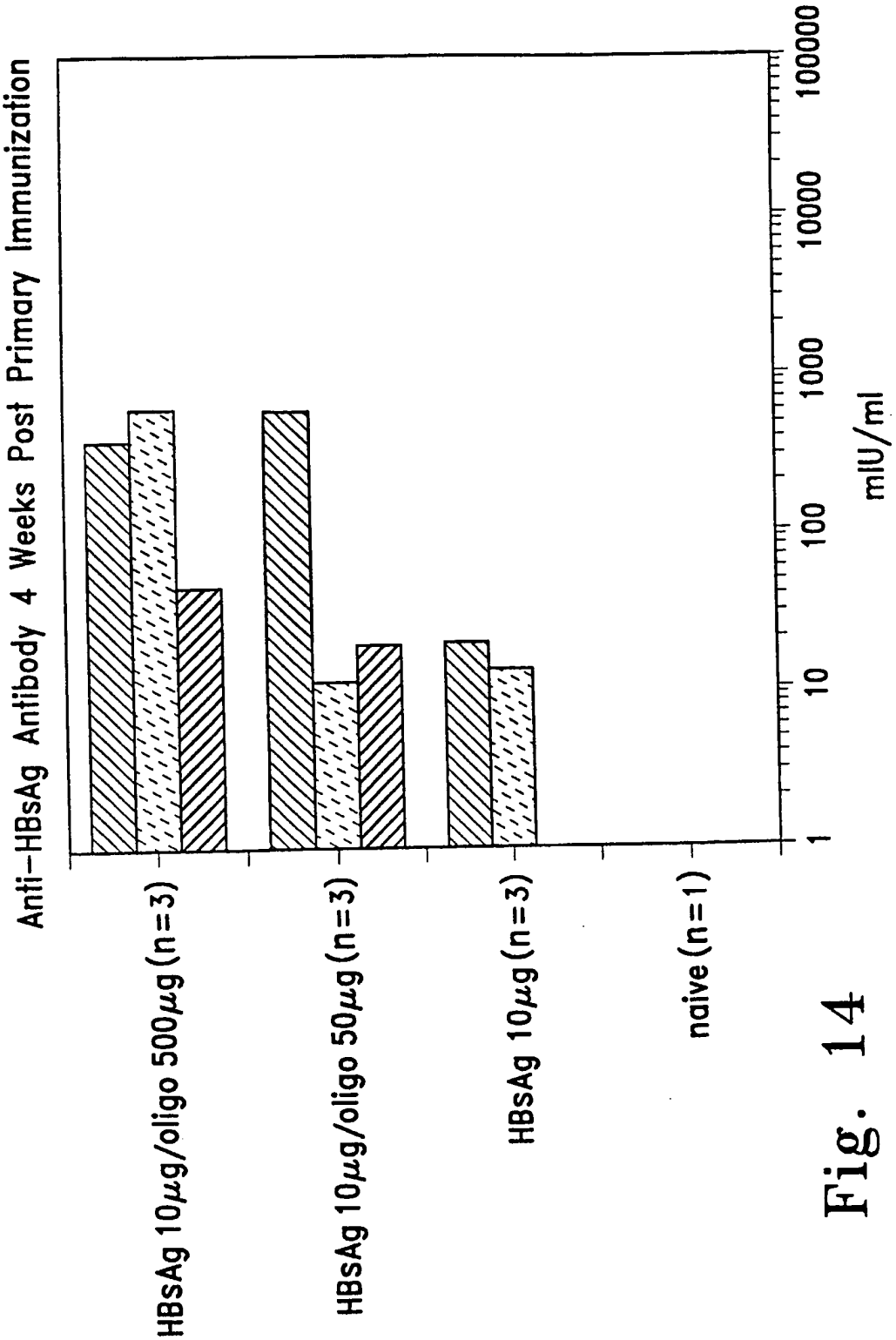
Anti-HBsAg Antibody 4 Weeks Post Primary Immunization
mIU/ml
1
10
100
1000
10000
100000
HBsAg 10μg/oligo 500μg (n=3)
HBsAg 10μg/oligo 50μg (n=3)
HBsAg 10μg (n=3)
naive (n=1)

Fig. 14

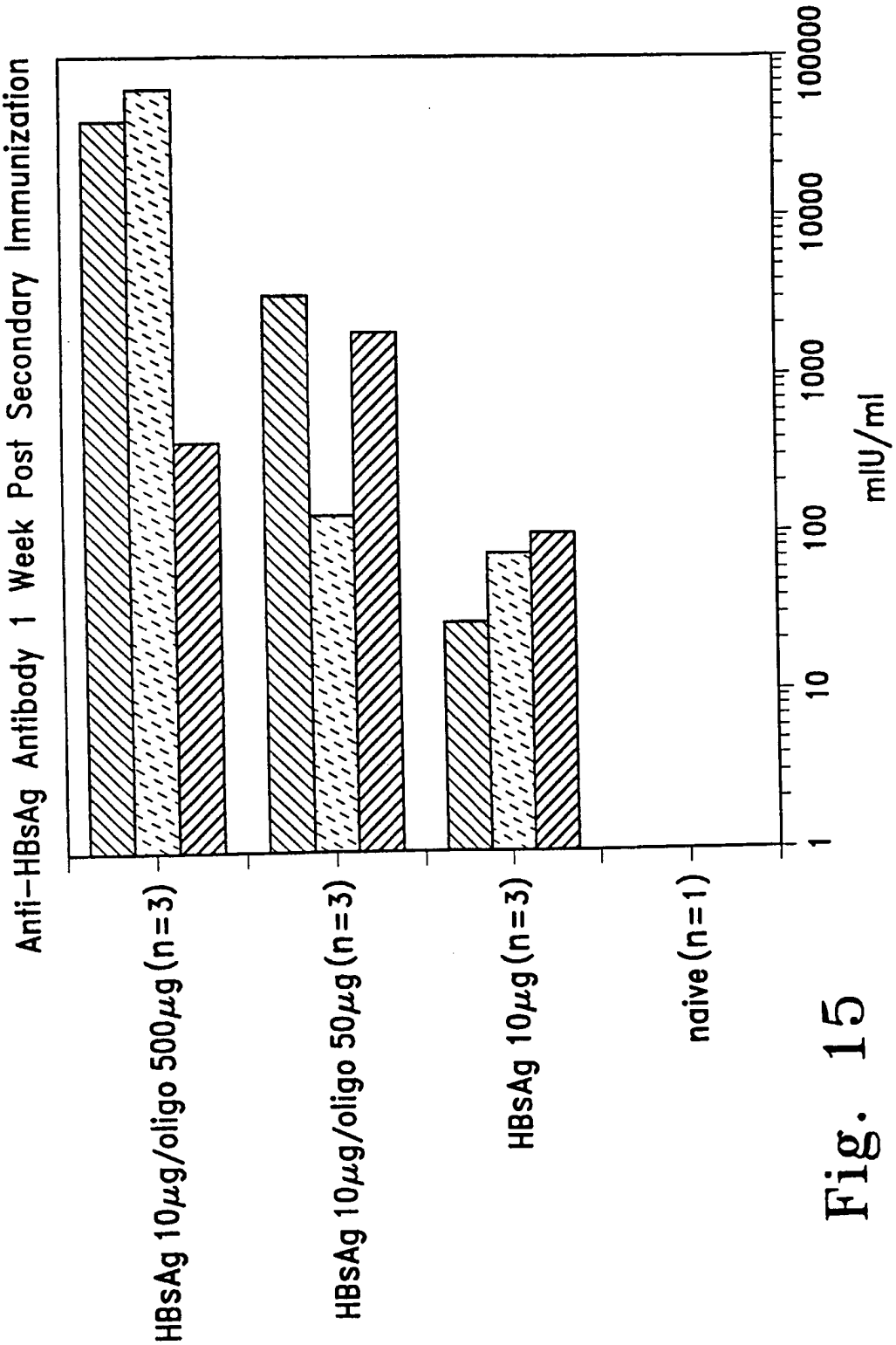
Anti-HBsAg Antibody 1 Week Post Secondary Immunization
HBsAg 10μg/oligo 500μg (n=3)
HBsAg 10μg/oligo 50μg (n=3)
HBsAg 10μg (n=3)
naive (n=1)
1
10
100
1000
10000
100000
mIU/ml

Fig. 15

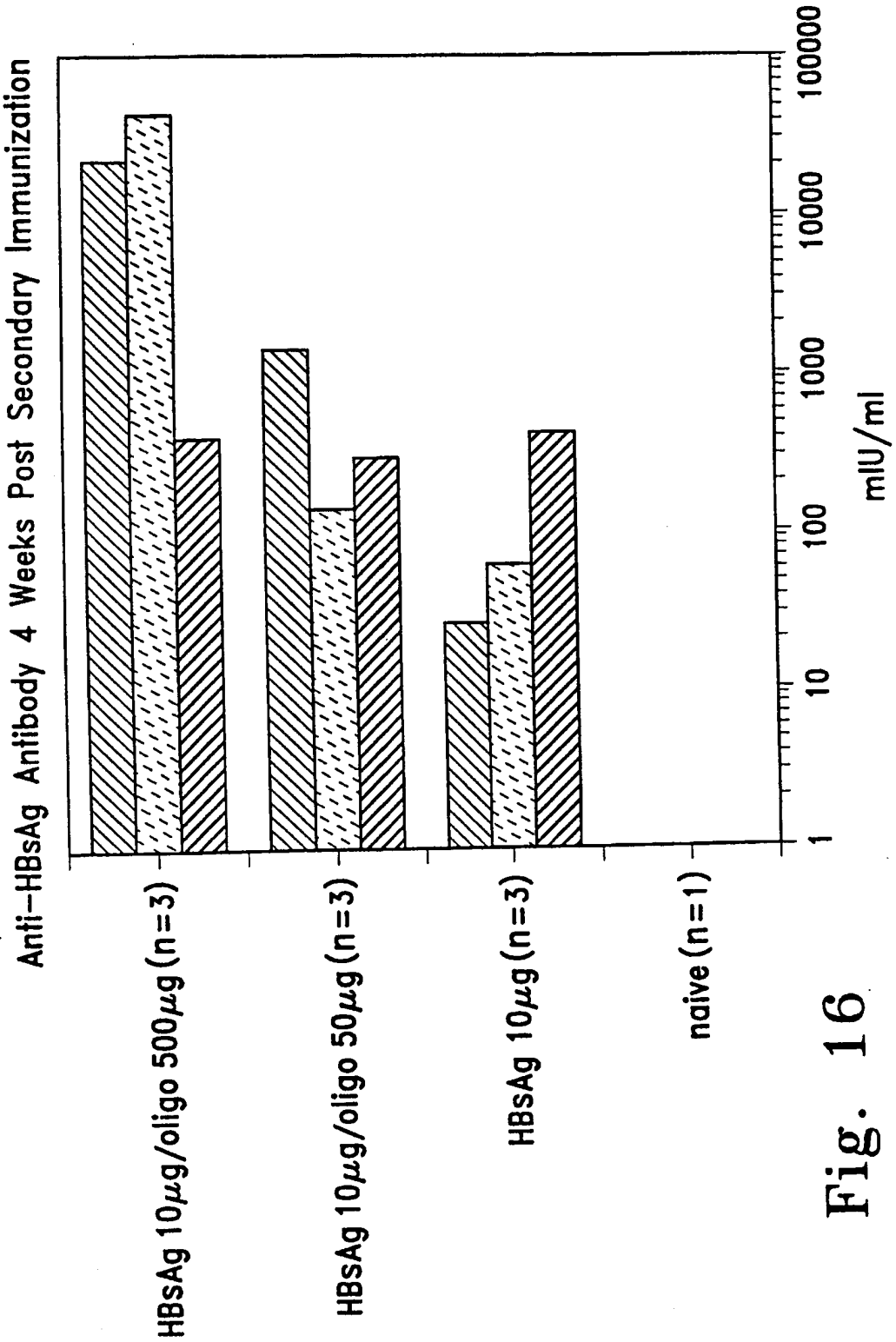
Anti-HBsAg Antibody 4 Weeks Post Secondary Immunization
mIU/ml
1
10
100
1000
10000
100000
HBsAg 10μg/oligo 500μg (n=3)
HBsAg 10μg/oligo 50μg (n=3)
HBsAg 10μg (n=3)
naive (n=1)

Fig. 16

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

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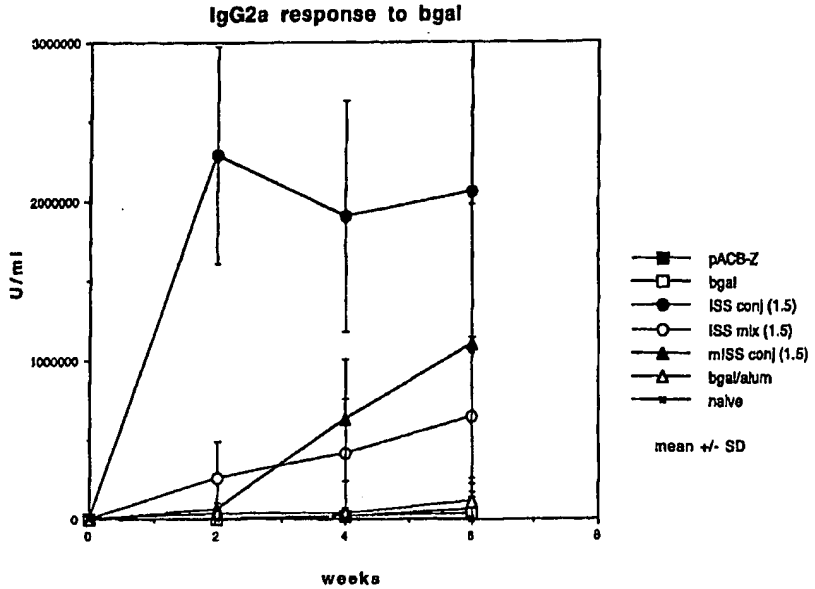

(57) Abstract

Immunostimulatory polynucleotide-immunomodulatory molecule conjugate compositions are disclosed. These compositions include a polynucleotide that is linked to an immunomodulatory molecule, which molecule comprises an antigen and may further comprise immunomodulators such as cytokines and adjuvants. The polynucleotide portion of the conjugate includes at least one immunostimulatory oligonucleotide nucleotide sequence (ISS). Methods of modulating an immune response upon administration of the polynucleotide-immunomodulatory conjugate preparation to a vertebrate host are also disclosed.

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IMMUNOSTIMULATORY POLYNUCLEOTIDE/IMMUNOMODULATORY MOLECULE CONJUGATES

RELATED U.S. PATENT APPLICATIONS

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FIELD OF THE INVENTION

The invention relates to compositions comprising an immunomodulatory molecule (IMM) including an antigen, conjugated to a polynucleotide that contains or consists of at least one immunostimulatory oligonucleotide (ISS-PN). It also relates to methods for modulating the immune response of a vertebrate host to an antigen.

HISTORY OF THE RELATED ART

Conventionally, immunization of a host against an antigen is accomplished by repeatedly vaccinating the host with the antigen. While most current vaccines elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex (MHC) class I-restricted cytotoxic T cells) are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, humoral responses are of little protective value against infection.

Given the weak cellular immune response to protein antigens, modulation of the immune responses to these antigens has clear importance. The ability to modify immune responses to protein or peptide antigen has implications for tumor therapy, for the treatment of allergic disorders and for treatment of other conditions achievable through induction of a vigorous cellular immune response.

SUMMARY OF THE INVENTION

The present invention provides compositions comprising an ISS-PN which is conjugated to an IMM (which includes an antigen) to form ISS-PN/IMM conjugates. The ISS-PN/IMM conjugates of the invention are biological response modifiers in the sense that they modify the humoral and cellular immune response of a host to an antigen.

Specifically, the ISS-PN and IMM components of the ISS-PN/IMM conjugates synergistically boost the magnitude of the host immune response against an antigen to a level greater than the host immune response to either the IMM, antigen or ISS-PN alone. The ISS-PN/IMM conjugates also shift the host cellular immune response away from the helper T lymphocyte type 2 (Th2) phenotype toward a helper T lymphocyte type 1 (Th1) phenotype. These responses to ISS-PN/IMM conjugates are particularly acute during the important early phase of the host immune response to an antigen.

To these ends, ISS-PN/IMM conjugates are delivered by any route through which antigen-sensitized host tissues will be contacted with the ISS-PN/IMM conjugate. ISS-PN/IMM conjugates administered in this fashion boost both humoral (antibody) and cellular (Th1 type) immune responses of the host. Thus, use of the method to boost the immune responsiveness of a host to subsequent challenge by a sensitizing antigen without immunization avoids the risk of Th2-mediated, immunization-induced anaphylaxis by suppressing IgE production in response to the antigen challenge. An especially advantageous use for this aspect of the invention is treatment of localized allergic responses in target tissues where the allergens enter the body, such as the skin and mucosa.

Suppression of the Th2 phenotype according to the invention is also a useful in reducing antigen-stimulated IL-4 and IL-5 production. Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to suppress the Th2

phenotype associated with conventional antigen immunization (e.g., for vaccination or allergy immunotherapy).

The shift to a Th1 phenotype achieved according to the invention is accompanied by increased secretion of IFN α, β and γ, as well as IL-12 and IL-18. Each of these cytokines enhance the host's immune defenses against intracellular pathogens, such as viruses. Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to combat pathogenic infection.

Angiogenesis is also enhanced in the Th1 phenotype (ostensibly through stimulation by IL-12). Thus, the invention encompasses delivery of ISS-PN/IMM conjugates to a host to stimulate therapeutic angiogenesis to treat conditions in which localized blood flow plays a significant etiological role; e.g., retinopathies.

The ISS-PN/IMM conjugates of the invention comprise an IMM conjugated to a polynucleotide that includes, or consists of, at least one immunostimulatory oligonucleotide (ISS-ODN) moiety. The ISS-ODN moiety is a single- or double-stranded DNA or RNA oligonucleotide having at least 6 nucleotide bases which may include, or consist of, a modified oligonucleoside or a sequence of modified nucleosides.

The ISS-ODN moieties comprise, or may be flanked by, a CpG containing nucleotide sequence or a p(IC) nucleotide sequence, which may be palindromic. Where the oligonucleotide moiety comprises a CpG sequence, it may include a hexamer structure consisting of: 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'. Examples of such hexamer structures are AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

In one aspect of the invention, the ISS-PN consists of an ISS-ODN. Alternatively, the ISS-PN comprises an ISS-ODN.

Conjugates of the invention also include PN/IMM wherein the PN serves as a carrier to introduce the IMM antigen into MHC Class I processing pathways not normally stimulated by soluble antigen, but lacks ISS activity and therefore does not stimulate a Th1 phenotype immune response. Examples of such PN/IMM are those wherein the CpG motif is mutated, for example, to a GpG motif.

In one aspect of the invention, the IMM conjugate partner to the ISS-PN consists of an antigen. Such antigens are selected from the group of antigens consisting of proteins, peptides, glycoproteins, polysaccharides and gangliosides.

In another aspect of the invention, the IMM conjugate partner comprises an antigen and further comprises an immunostimulatory molecule selected from the group of such molecules consisting of adjuvants, hormones, growth factors, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

In another aspect of the invention, the ISS-PN/IMM conjugate is modified for targeted delivery by, for example, attachment to a monoclonal antibody, receptor ligand and/or liposome.

Pharmaceutically acceptable compositions of ISS-PN/IMM conjugates are provided for use in practicing the methods of the invention. Where appropriate to the contemplated course of therapy, the ISS-PN/IMM conjugates may be administered with anti-inflammatory or immunotherapeutic agents. Thus, a particularly useful composition for use in practicing the method of the invention is one in which an anti-inflammatory agent (e.g., a glucocorticoid) is mixed with, or further conjugated to, an ISS-PN/IMM conjugate.

The ISS-PN/IMM conjugates can also be provided in the form of a kit comprising ISS-PN/IMM conjugates and any additional medicaments, as well as a device for delivery of the ISS-PN/IMM conjugates to a host tissue and reagents for determining the biological effect of the ISS-PN/IMM conjugates on a treated host.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM (1:5 ratio) in comparison to the levels of Th2-like responses stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG2a levels in naive (unexposed) mice. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 2 is a graph of data demonstrating the levels of Th2-type immune responses (as measured by production of IgG1 against an IMM antigen) stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS (1:5 ratio); the antigen conjugated to a non-stimulatory PN (mISS conj; 1:5 ratio); the antigen in adjuvant (alum) and, for reference, the IgG1 levels in naive (unexposed) mice, all as compared to the vigorous Th1-type immune response produced in mice immunized with ISS-PN/IMM (1:5 ratio). The horizontal axis represents the levels (units/ml) of antibody; the vertical axis represents the number of weeks following primary antigen exposure.

FIGURE 3 is a graph of data demonstrating the vigorous Th1-type immune response (as measured by production of IgG2a against an IMM antigen) stimulated by ISS-PN/IMM in comparison to the levels of Th2-like responses stimulated by the antigen alone (AgE) and antigen conjugated to a non-stimulatory PN (mISS conj). Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 4 is a graph of data demonstrating the levels of Th2-type immune responses (as measured by production of IgG1 against an IMM antigen) stimulated by the antigen alone (AgE) and antigen conjugated to a non-stimulatory PN (mISS conj) in comparison to the vigorous Th1-type immune response stimulated in ISS-PN/IMM immunized mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (units/ml) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 5 is a graph of data demonstrating suppression of Th2 associated anti-antigen (AgE) IgE production by ISS-PN/IMM in comparison to the levels of IgE production stimulated by the antigen alone (AgE) and the antigen conjugated to a non-stimulatory PN (mISS conj). Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (counts per minute; cpm) of antibody; the vertical axis shows the levels at 4 weeks following primary antigen exposure (shaded bars) and at 2 weeks following secondary antigen challenge (solid bars).

FIGURE 6 is a graph of data demonstrating the high levels of Th1 associated interferon γ (IFNg) production stimulated by ISS-PN/IMM in comparison to the relatively low levels of the Th1 cytokine stimulated by an ISS containing, antigen encoding plasmid (pACB-Z); the antigen alone (β-gal); the antigen mixed with an ISS; the antigen conjugated to a non-stimulatory PN (mISS conj); the antigen in adjuvant (alum) and, for reference, the IFNg levels in naive (unexposed) mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels (ng/ml) of cytokine; the vertical axis shows the levels of cytokine at 4 weeks following primary antigen exposure (shaded bars).

FIGURE 7 is a graph of data demonstrating the vigorous antigen-specific cytotoxic T lymphocyte (CTL) response stimulated by ISS-PN/IMM in comparison to the levels of CTL production stimulated by an ISS containing, antigen encoding plasmid (pACB-

Z); the antigen alone (β-gal); the antigen mixed with an ISS; the antigen conjugated to a non-stimulatory PN (mISS conj); the antigen in adjuvant (alum) and, for reference, the CTL levels in naive (unexposed) mice. Antigen to PN ratios are all 1:5. The horizontal axis represents the levels of antigen-specific cell lysis obtained (as a percentage of control; no antigen); the vertical axis shows the levels of CTL detected at different effector (antigen) to target ratios, from 0:1 to 10:1. The legend identifies how each cell population was treated.

DETAILED DESCRIPTION OF THE INVENTION

A. *Biological Activity of the ISS-PN/IMM Conjugates*

The immune response stimulated by the ISS-PN/IMM conjugates of the invention differs from the vertebrate immune response to conventional vaccination in both magnitude and quality. In the former respect, the host immune response to an antigen is boosted to a level greater than achieved on exposure to an ISS-PN or antigen administered alone or together in an unconjugated form. Thus, one surprising aspect of the invention is that conjugation of an ISS-PN to an antigen-containing IMM produces a synergism between the immunostimulatory activity of the ISS-PN and the immunomodulatory activity of the IMM that immunizes the host to the antigen more effectively than one would predict.

Advantageously, the immune response stimulated according to the invention differs from the immune response of vertebrates to conventional vaccination in that the latter develops in a Th2 phenotype while the former develops in a Th1 phenotype. In this regard, it is helpful to recall that CD4+ lymphocytes generally fall into one of two distinct subsets; i.e., the Th1 and Th2 cells. Th1 cells principally secrete IL-2, IFNγ and TNFβ (the latter two of which mediate macrophage activation and delayed type hypersensitivity) while Th2 cells principally secrete IL-4 (which stimulates production of IgE antibodies), IL-5 (which stimulates granulocyte infiltration of tissue), IL-6 and IL-10. These CD4+ subsets exert a negative influence on one another; i.e., secretion of Th1 lymphokines inhibits secretion of Th2 lymphokines and vice versa.

Factors believed to favor Th1 activation resemble those induced by viral infection and include intracellular pathogens, exposure to IFN-β, IFN-α, IFNγ, IL-12 and IL-18 and exposure to low doses of antigen. Th1 type immune responses also predominate in autoimmune disease. Factors believed to favor Th2 activation include exposure to IL-4 and IL-10, APC activity on the part of B lymphocytes and high doses of antigen.

Active Th1 (IFNγ) cells enhance cellular immunity and are therefore of particular value in responding to intracellular infections, while active Th2 cells enhance antibody production and are therefore of value in responding to extracellular infections (at the risk of anaphylactic events associated with IL-4 stimulated induction of IgE antibody production). Thus, the ability to shift host immune responses from the Th1 to the Th2 repertoire and vice versa has substantial clinical significance for controlling host immunity against antigen challenge (e.g., in infectious and allergic conditions).

To that end, the methods of the invention shift the host immune response to a sensitizing antigen toward a Th1 phenotype (Example I). Consequently, Th2 associated cytokine production and antigen stimulated production of IgE (Examples II and III) are suppressed, thereby reducing the host's risk of prolonged allergic inflammation and minimizing the risk of antigen-induced anaphylaxis. CTL production is also stimulated to a greater degree in animals treated according to the invention. Because CTL production is tied to antigen processing in Class I MHC pathways, increased CTL production can be produced from non-immunostimulatory PN/IMM as well as ISS-PN/IMM (Example IV).

Although the invention is not limited to any particular mechanism of action, it is conceivable that PN facilitate uptake of exogenous antigen by antigen presenting cells for presentation through host MHC Class I processing pathways not normally stimulated by soluble antigen. Thus, ISS-PN/IMM carry antigen into MHC Class I processing pathways (which may also be achieved by PN/IMM without ISS activity) then stimulate a cytokine cascade in a Th1 phenotype (as a result of ISS activity). Whatever the mechanism of action, use of ISS-PN/IMM to boost the host's immune responsiveness to a sensitizing antigen and shift the immune response toward a Th1 phenotype avoids the risk of immunization-induced anaphylaxis, suppresses IgE production in response to a sensitizing antigen and eliminates the need to identify the sensitizing antigen for use in immunization.

With reference to the invention, "boosting of immune responsiveness in a Th1 phenotype" in an ISS-PN/IMM treated host is evidenced by:

(1) a reduction in levels of IL-4 measured before and after antigen-challenge; or detection of lower (or even absent) levels of IL-4 in a treated host as compared to an antigen-primed, or primed and challenged, control;

(2) an increase in levels of IL-12, IL-18 and/or IFN (α, β or γ) before and after antigen challenge; or detection of higher levels of IL-12, IL-18 and/or IFN (α, β or γ) in an ISS-PN/IMM treated host as compared to an antigen-primed or, primed and challenged, control;

(3) IgG2a antibody production in a treated host; or

(4) a reduction in levels of antigen-specific IgE as measured before and after antigen challenge; or detection of lower (or even absent) levels of antigen-specific IgE in an ISS-PN/IMM treated host as compared to an antigen-primed, or primed and challenged, control.

Exemplary methods for determining such values are described further in the Examples.

Thus, the ISS-PN/IMM conjugates of the invention provide relatively safe, effective means of stimulating a robust immune response in a vertebrate host against any antigen.

B. *ISS-PN/IMM Conjugates: Structure and Preparation*

1. ISS-PN root structure

The ISS-ODN base of the ISS-PN/IMM conjugates of the invention includes an oligonucleotide, which may be a part of a larger nucleotide construct such as a

plasmid. The term "polynucleotide" therefore includes oligonucleotides, modified oligonucleotides and oligonucleosides, alone or as part of a larger construct. The polynucleotide may be single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA).

The polynucleotide portion can be linearly or circularly configured, or the oligonucleotide portion can contain both linear and circular segments. Modifications of oligonucleotides include, but are not limited to, modifications of the 3'OH or 5'OH group, modifications of the nucleotide base, modifications of the sugar component, and modifications of the phosphate group.

The oligonucleotide base of ISS-PN/IMM conjugates may comprise ribonucleotides (containing ribose as the only or principal sugar component), deoxyribonucleotides deoxyribose as the principal sugar component), or in accordance with established state-of-the-art modified sugars or sugar analogs may be incorporated in the oligonucleotide of the present invention. Thus, in addition to ribose and deoxyribose, the sugar moiety may be pentose, deoxypentose, hexose, deoxyhexos, glucose, arabinose, xylose, lyxose, and a sugar "analog" cyclopentyl group. The sugar may be in pyranosyl or in a furanosyl form. In the modified oligonucleotides of the present invention the sugar moiety is preferably the furanoside of ribose, deoxyribose, arabinose or 2'-0-methylribose, and the sugar may be attached to the respective heterocyclic bases either in I or J anomeric configuration. The preparation of these sugars or sugar analogs and the respective "nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) per se is known, and need not be described here, except to the extent such preparation may pertain to any specific example.

The phosphorous derivative (or modified phosphate group) which may be attached to the sugar or sugar analog moiety in the modified oligonucleotides of the present invention may be a monophosphate, diphosphate, triphosphate, alkylphosphate,

alkanephosphate, phosphoronthioate, phosphorodithioate or the like. The preparation of the above-noted phosphate analogs, and their incorporation into nucleotides, modified nucleotides and oligonucleotides, per se, is also known and need not be described here.

The heterocyclic bases, or nucleic acid bases which are incorporated in the oligonucleotide base of the ISS-PN/IMM conjugates may be the naturally occurring principal purine and pyrimidine bases, (namely uracil or thymine, cytosine, adenine and guanine, as mentioned above), as well as naturally occurring and synthetic modifications of said principal bases. Those skilled in the art will recognize that a large number of "synthetic" non-natural nucleosides comprising various heterocyclic bases and various sugar moieties (and sugar analogs) have become available in the prior art, such that oligonucleotide base of the ISS-PN/IMM conjugates may include one or several heterocyclic bases other than the principal five base components of naturally occurring nucleic acids. Preferably, however, the heterocyclic base in the oligonucleotide base of the ISS-PN/IMM conjugates is selected form uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4-aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrolo [2,3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the oligonucleotides via the 9-position, the pyrimidines via the 1-position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

Structurally, the root oligonucleotide of the ISS-PN component of ISS-PN/IMM is a non-coding sequence which may include at least one unmethylated CpG motif. The relative position of any CpG sequence in ISS-PN with immunostimulatory activity in certain mammalian species (e.g., rodents) is 5'-CG-3' (i.e., the C is in the 5' position with respect to the G in the 3' position). PN/IMM can be conveniently obtained by substituting the cytosine in the CpG dinucleotide with another nucleotide; a particularly useful substitution is with a guanine to form GpG dinucleotide containing PN.

Some oligonucleotide ISS (ISS-ODN) are known. In such ISS-ODN, the CpG motif is flanked by at least two purine nucleotides (e.g., GA or AA) and at least two pyrimidine nucleotides (5'-Purine-Purine-[C]-[G]-Pyrimidine-Pyrimidine-3'). CpG motif-containing ISS-ODN are believed to stimulate B lymphocyte proliferation (see, e.g., Krieg, *et al.*, *Nature*, 374:546-549, 1995).

The core hexamer structure of the foregoing ISS-PN may be flanked upstream and/or downstream by any number or composition of nucleotides or nucleosides. However, ISS-PN are at least 6 bases in length, and preferably are between 6 and 200 bases in length, to enhance uptake of the ISS-PN/IMM into target tissues. Those of ordinary skill in the art will be familiar with, or can readily identify, reported nucleotide sequences of known ISS-ODN for reference in preparing ISS-PN. For ease of reference in this regard, the following sources are especially helpful:

Yamamoto, *et al.*, *Microbiol.Immunol.*, 36:983 (1992)
Ballas, *et al.*, *J.Immunol.*, 157:1840 (1996)
Klinman, *et al.*, *J.Immunol.*, 158:3635 (1997)
Sato, *et al.*, *Science*, 273:352 (1996)

Each of these articles are incorporated herein by reference for the purpose of illustrating the level of knowledge in the art concerning the nucleotide composition of known ISS-ODN .

In particular, ISS-PN and PN useful in the invention include those which have the following hexameric nucleotide sequences:

1. For ISS-PN, hexamers having "CpG" motifs or, for PN, hexamers having XpY motifs, where X cannot be C if Y is G and vice-versa; and,

2. Inosine and/or uracil substitutions for nucleotides in the foregoing hexamer sequences for use as RNA ISS-ODN.

For example, DNA based ISS-PN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGTT, AGCGTC, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AGCGCT, GACGCT, GGCGCT, TTCGAA, GGCGTT and AACGCC (respectively, SEQ.ID.Nos. 1-18).

RNA based ISS-PN useful in the invention include those which have the following hexameric nucleotide sequences:

AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC, and poly(I•C) (respectively, SEQ.ID.Nos. 19-33).

The ISS-PN may or may not include palindromic regions. If present, a palindrome may extend only to a CpG motif, if present, in the core hexamer sequence, or may encompass more of the hexamer sequence as well as flanking nucleotide sequences.

In addition, backbone phosphate group modifications (e.g., methylphosphonate, phosphorothioate, phosphoroamidate and phosphorodithioate internucleotide linkages) can confer anti-microbial activity on the ISS-PN and enhance their stability *in vivo*, making them particularly useful in therapeutic applications. A particularly useful phosphate group modification is the conversion to the phosphorothioate or phosphorodithioate forms of ISS-PN. In addition to their potentially anti-microbial

properties, phosphorothioates and phosphorodithioates are more resistant to degradation *in vivo* than their unmodified oligonucleotide counterparts, making the ISS-PN/IMM of the invention more available to the host.

2. IMM conjugate partners.

The oligonucleotide base of the ISS-PN/IMM conjugate is conjugated to an IMM which includes an antigen and may further include an immunomodulatory agent. An "antigen" is a substance that is recognized and bound specifically by an antibody or by a T cell antigen receptor. Antigens can include peptides, proteins, glycoproteins and polysaccharides, including portions thereof and combinations thereof. The antigens can be those found in nature or can be synthetic.

The term "immunomodulatory" as used herein includes immunostimulatory as well as immunosuppressive effects. Immunostimulatory effects include, but are not limited to, those that directly or indirectly enhance cellular or humoral immune responses. Examples of immunostimulatory effects include, but are not limited to, increased antigen-specific antibody production; activation or proliferation of a lymphocyte population such as NK cells, CD4+ T lymphocytes, CD8+ T lymphocytes, macrophages and the like; as well as increased synthesis of Th1 associated immunostimulatory cytokines including, but not limited to, IL-6, IL-12, IL-18, IFN-α,β and γ, TNF-α and the like. Immunosuppressive effects include those that directly or indirectly decrease cellular or humoral immune responses.

Examples of immunosuppressive effects include, but are not limited to, a reduction in antigen- specific antibody production such as reduced IgE production; activation of lymphocyte or other cell populations that have immunosuppressive activities such as those that result in immune tolerance; and increased synthesis of cytokines that have suppressive effects toward certain cellular functions. One example of this is IFN-γ, which can block IL-4 induced class switch to IgE and IgG1, thereby reducing the

levels of these antibody subclasses.

Thus, an "immunomodulatory agent" suitable for use as conjugate partners for ISS-PN/IMM can be a peptide, such as an antigen or cytokine. Where the ISS-PN/IMM conjugate partner is a peptide, suitable peptides include purified native peptides, synthetic peptides, recombinant proteins, crude protein extracts, attenuated or inactivated viruses, cells, micro-organisms, or fragments of such peptides.

Protein antigens that can serve as IMM conjugate partners include antigens from a wide variety of sources, including allergens such as plant pollens, dust mite proteins, animal dander, saliva, and fungal spores as well as infectious microorganims. Examples of the latter include attenuated or inactivated viruses such as HIV-1, HIV-2, hepatitis, herpes simplex, rotavirus, polio virus, measles virus, human and bovine papilloma virus, and slow brain viruses. For immunization against tumor formation, the conjugate can include tumor cells (live or irradiated), tumor cell extracts, or protein subunits of tumor antigens. Vaccines for immuno-based contraception can be formed by including sperm proteins as the peptide portion of the conjugate.

Among the suitable cytokines for use as components of IMM conjugate partners are the interleukins (IL-1, IL-2, IL-3, etc.), interferons (e.g., IFN-α, IFN-β, IFN-γ), erythropoietin, colony stimulating factors (e.g., G-CSF, M-CSF, GM-CSF) and TNF-α.

IMM conjugate partners can also include amino acid sequences that mediate protein binding to a specific receptor or that mediate targeting to a specific cell type or tissue. Examples include, but are not limited to, antibodies or antibody fragments; peptide hormones such as human growth hormone; and enzymes. Co-stimulatory molecules such as B7 (CD80), trans-activating proteins such as transcription factors, chemokines such as macrophage chemotactic protein (MCP) and other chemoattractant or chemotactic peptides are also useful peptide-based conjugate partners.

More specifically, suitable antigens for use as ISS-PN/IMM conjugate partners include any molecule capable of being conjugated to an oligonucleotide and eliciting a B cell or T cell antigen-specific response. Preferably, antigens elicit an antibody response specific for the antigen. A wide variety of molecules are antigens. These include, but are not limited to, sugars, lipids, autacoids and hormones, as well as macromolecules such as complex carbohydrates, and phospholipids. Small molecules may need to be haptenized in order to be rendered antigenic.

Preferably the antigens are peptides, polysaccharides (such as the capsular polysaccharides used in *Haemophilus influenza* vaccines), gangliosides and glycoproteins. The antigen may be an intact antigen or T cell epitope(s) of an antigen. These can be obtained through several methods known in the art, including isolation and synthesis using chemical and enzymatic methods. In certain cases, such as for many sterols fatty acids and phospholipids, the antigenic portions are commercially available.

Many antigenic peptides and proteins are known in, and available to the art; others can be identified using conventional techniques. Examples of known antigens include, but are not limited to :

a. Allergens such as reactive major dust mite allergens *Der pI* and *Der pII* (*see,* Chua, *et al., J.Exp.Med.*, 167:175-182, 1988; and, Chua, *et al., Int.Arch.Allergy Appl. Immunol.*, 91:124-129, 1990), T cell epitope peptides of the *Der pII* allergen (*see*, Joost van Neerven, *et al., J.Immunol.*, 151:2326-2335, 1993), the highly abundant Antigen E (*Amb aI*) ragweed pollen allergen (*see,* Rafnar, *et al., J.Biol.Chem.*, 266:1229-1236, 1991), phospholipase A_2 (bee venom) allergen and T cell epitopes therein (*see,* Dhillon, *et al., J.Allergy Clin.Immunol.*, __:42-__, 1992), white birch pollen (*Betvl)* (*see,* Breiteneder, *et al., EMBO,* 8:1935-1938, 1989), the *Fel dI* major domestic cat allergen (*see,* Rogers, *et al., Mol.Immunol.*, 30:559-568, 1993), tree pollen (*see*, Elsayed et al., *Scand. J. Clin. Lab. Invest. Suppl.*, 204:17-31,

1991) and grass pollen (*see*, Malley, *J. Reprod. Immunol.*, 16:173-86, 1989).

b. Live, attenuated and inactivated microorganisms such as inactivated polio virus (Jiang *et al.*, *J. Biol. Stand.*, 14:103-9, 1986), attenuated strains of Hepatitis A virus (Bradley *et al.*, *J. Med. Virol.*, 14:373-86, 1984), attenuated measles virus (James *et al.*, *N. Engl. J. Med.*, 332:1262-6, 1995) and epitopes of pertussis virus (e.g., ACEL-IMUNE® acellular DTP, Wyeth-Lederle Vaccines and Pediatrics).

c. Contraceptive antigens such as human sperm protein (Lea *et al.*, *Biochim. Biophys. Acta*, 1307:263, 1996).

The published sequence data and methods for isolation and synthesis of the antigens described in these articles are incorporated herein by this reference to illustrate knowledge in the art regarding useful antigen sources. Those of ordinary skill in the art will be familiar with, or can readily ascertain, the identity of other useful antigens for use as ISS-PN/IMM conjugate partners.

Particularly useful immunostimulatory peptides for inclusion in IMM are those which stimulate Th1 immune responses, such as IL-12 (Bliss, *et al.*, *J.Immunol.*, 156:887-894, 1996), IL-18, INF-α,β and γ or TGF-α. Conjugation of adjuvants (such as keyhole limpet hemocyanin, KLH) to the ISS-PN/IMM conjugate can further enhance the activity of the ISS-PN/IMM conjugates of the invention.

Other useful adjuvants include cholera toxin, procholeragenoid, cholera toxin B subunit and fungal polysaccharides including, but not limited to, schizophyllan, muramyl dipeptide, muramyl dipeptide derivatives, phorbol esters, microspheres, non-Helicobacter pylori bacterial lysates, labile toxin of Escherichia coli, block polymers, saponins, and ISCOMs. For additional adjuvants, those of ordinary skill in the art may also refer to, for example, Azuma, I., "Synthetic Immunoadjuvants: Application to Non-Specific Host Stimulation and Potentiation of Vaccine Immunogenicity"

Vaccine, vol. 10, 1000 (1992); Pockley, A.G. & Montgomery, P.C., "In vivo Adjuvant Effect of Interleukins 5 and 6 on Rat Tear IgA Antibody Responses" *Immunology*, vol. 73, 19-23 (1991); Adam, A. & Lederer, E. "Muramyl peptides as Immunomodulators" ISI ATLAS OF SCIENCE 205 (1988); Clements, J.D., et al. "Adjuvant Activity of Escherichia coli Heat-labile Enterotoxin and Effect on the Induction of Oral Tolerance in Mice to Unrelated Protein Antigens" *Vaccine*, vol. 6, 269 (1988); Ben Ahmeida, E.T.S., *et al.* "Immunopotentiation of Local and Systemic Humoral Immune Responses by ISCOMs, Liposomes and FCA: Role in Protection Against Influenza A in Mice" *Vaccine*, vol. 11, 1302 (1993); and Gupta, R.K. *et al.* "Adjuvants -- A Balance Between Toxicity and Adjuvanticity" *Vaccine*, vol. 11, 290-308 (1993).

Those of ordinary skill in the art will appreciate that non-antigen components of IMM described above can also be administered in unconjugated form with an ISS-PN/IMM (antigen only) conjugate. Thus, the co-administration of such components is encompassed by the invention.

C. *Synthesis of Polynucleotide Conjugates*

1. Polynucleotide portion

ISS-PN can be synthesized using techniques and nucleic acid synthesis equipment which are well-known in the art. For reference in this regard, see, e.g., Ausubel, *et al.*, *Current Protocols in Molecular Biology,* Chs. 2 and 4 (Wiley Interscience, 1989); Maniatis, *et al., Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., New York, 1982); U.S. Patent No. 4,458,066 and U.S. Patent No. 4,650,675. When assembled enzymatically, the individual units can be ligated with a ligase such as T4 DNA or RNA ligase as described in, for example, U.S. Patent No. 5,124,246. Oligonucleotide degradation could be accomplished through the exposure of an oligonucleotide to a nuclease, as exemplified in U.S. Patent No. 4,650,675. These references are incorporated herein by reference for the sole purpose of demonstrating knowledge in the art concerning production of synthetic polynucleotides. Because the

ISS-PN is non-coding, there is no concern about maintaining an open reading frame during synthesis.

Alternatively, ISS-PN may be isolated from microbial species (especially mycobacteria) using techniques well-known in the art, such as nucleic acid hybridization. Preferably, such isolated ISS-PN will be purified to a substantially pure state; i.e., to be free of endogenous contaminants, such as lipopolysaccharides. ISS-PN isolated as part of a larger polynucleotide can be reduced to the desired length by techniques well known in the art, such as by endonuclease digestion. Those of ordinary skill in the art will be familiar with, or can readily ascertain, techniques suitable for isolation, purification and digestion of polynucleotides to obtain ISS-PN of potential use in the invention.

Circular ISS-PN can be isolated, synthesized through recombinant methods, or chemically synthesized. Where the circular ISS-PN is obtained through isolation or through recombinant methods, the ISS-PN will preferably be a plasmid. The chemical synthesis of smaller circular oligonucleotides can be performed using literature methods (Gao et al., Nucleic Acids Res. (1995) 23:2025-9; Wang et al., Nucleic Acids Res. (1994) 22:2326-33).

The ISS-PN can also contain modified oligonucleotides. These modified oligonucleotides can be synthesized using standard chemical transformations. The efficient solid-support based construction of methylphosphonates has been described. Agrawal et al. (19) Tet. Lett. 28:3539-3542. The synthesis of other phosphorous based modified oligonucleotides, such as phosphotriesters (Miller et al. JACS 93, 6657-6665), phosphoramidates (Jager et al, Biochemistry 27, 7247-7246), and phosphorodithioates (U.S. Patent No. 5,453,496) has also been described. Other non-phosphorous based modified oligonucleotides can also be used (Stirchak et al., Nucleic Acids Res. 17, 6129-6141).

The preparation of base-modified nucleosides, and the synthesis of modified oligonucleotides using said base-modified nucleosides as precursors, has been described, for example, in U.S. Patents 4,910,300, 4,948,882, and 5,093,232. These base-modified nucleosides have been designed so that they can be incorporated by chemical synthesis into either terminal or internal positions of an oligonucleotide. Such base-modified nucleosides, present at either terminal or internal positions of an oligonucleotide, can serve as sites for attachment of a peptide or other antigen. Nucleosides modified in their sugar moiety have also bee described (e.g., U.S. Patents 4,849,513, 5,015,733, 5,118,800, 5,118,802) and can be used similarly.

The techniques for making phosphate group modifications to oligonucleotides are known in the art and do not require detailed explanation. For review of one such useful technique, the an intermediate phosphate triester for the target oligonucleotide product is prepared and oxidized to the naturally occurring phosphate triester with aqueous iodine or with other agents, such as anhydrous amines. The resulting oligonucleotide phosphoramidates can be treated with sulfur to yield phophorothioates. The same general technique (excepting the sulfur treatment step) can be applied to yield methylphosphoamidites from methylphosphonates. For more details concerning phosphate group modification techniques, those of ordinary skill in the art may wish to consult U.S. Patent Nos. 4,425,732; 4,458,066; 5,218,103 and 5,453,496, as well as *Tetrahedron Lett.* at 21:4149 (1995), 7:5575 (1986), 25:1437 (1984) and *Journal Am.ChemSoc.*, 93:6657 (1987), the disclosures of which are incorporated herein for the sole purpose of illustrating the standard level of knowledge in the art concerning preparation of these compounds.

2. Linking the PN component to the IMM component

The ISS-PN component can be linked to the IMM portion of the conjugate in a variety of ways. The link can be made at the 3' or 5' end of the ISS-PN, or to a suitably modified base at an internal position in the PN. If the peptide contains a suitable

reactive group (e.g., an N-hydroxysuccinimide ester) it can be reacted directly with the N^4 amino group of cytosine residues. Depending on the number and location of cytosine residues in the ISS-PN, specific labeling at one or more residues can be achieved.

Alternatively, modified oligonucleosides, such as are known in the art, can be incorporated at either terminus, or at internal positions in the ISS-PN. These can contain blocked functional groups which, when deblocked, are reactive with a variety of functional groups which can be present on, or attached to, a peptide of interest.

The IMM portion of the conjugate can be attached to the 3'-end of the ISS-PN through solid support chemistry. For example, the ISS-PN portion can be added to a polypeptide portion that has been pre-synthesized on a support (Haralambidis et al., Nucleic Acids Res. (1990) 18:493-99; Haralambidis et al., Nucleic Acids Res. (1990) 18:501-505). Alternatively, the PN can be synthesized such that it is connected to a solid support through a cleavable linker extending from the 3'-end. Upon chemical cleavage of the ISS-PN from the support, a terminal thiol group is left at the 3'-end of the ISS-PN (Zuckermann et al., Nucleic Acids Res. (1987) 15:5305-5321; Corey et al., (1987) Science 238:1401-1403), or a terminal amine group is left at the 3'-end of the PN (Nelson et al., Nucleic Acids Res. (1989) 17:1781-94). Conjugation of the amino-modified PN to amino groups of the peptide can be performed as described in Benoit et al., Neuromethods (1987) 6:43-72. Conjugation of the thiol-modified ISS-PN to carboxyl groups of the peptide can be performed as described in Sinah et al., Oligonucleotide Analogues: A Practical Approach (1991) IRL Press.

The IMM portion of the conjugate can be attached to the 5'-end of the ISS-PN through an amine, thiol, or carboxyl group that has been incorporated into the ISS-PN during its synthesis. Preferably, while the ISS- PN is fixed to the solid support, a linking group comprising a protected amine, thiol, or carboxyl at one end, and a phosphoramidite at the other, is covalently attached to the 5'-hydroxyl (Agrawal et al.,

Nucleic Acids Res. (1986) 14:6227-6245; Connolly, Nucleic Acids Res. (1985) 13:4485-4502; Coull et al., Tetrahedron Lett. (1986) 27:3991-3994; Kremsky et al., Nucleic Acids Res. (1987) 15:2891-2909; Connolly, Nucleic Acids Res. (1987) 15:3131-3139; Bischoff et al., Anal. Biochem. (1987) 164:336-344; Blanks et al., Nucleic Acids Res. (1988) 16:10283-10299; U.S. Patent Nos. 4,849,513, 5,015,733, 5,118,800, and 5,118,802). Subsequent to deprotection, the latent amine, thiol, and carboxyl functionalities can be used to covalently attach the PN to a peptide (Benoit et al., Neuromethods (1987) 6:43-72; Sinah et al., Oligonucleotide Analogues: A Practical Approach (1991) IRL Press).

A peptide portion can be attached to a modified cytosine or uracil at any position in the ISS-PN. The incorporation of a "linker arm" possessing a latent reactive functionality, such as an amine or carboxyl group, at C-5 of the modified base provides a handle for the peptide linkage (Ruth, 4th Annual Congress for Recombinant DNA Research, p. 123).

The linkage of the ISS-PN to a peptide can also be formed through a high-affinity, non-covalent interaction such as a biotin-streptavidin complex. A biotinyl group can be attached, for example, to a modified base of an oligonucleotide (Roget et al., Nucleic Acids Res. (1989) 17:7643-7651). Incorporation of a streptavidin moiety into the peptide portion allows formation of a non-covalently bound complex of the streptavidin conjugated peptide and the biotinylated PN.

The linkage of the ISS-PN to a lipid can be formed using standard methods. These methods include, but are not limited to, the synthesis of oligonucleotide-phospholipid conjugates (Yanagawa et al., Nucleic Acids Symp. Ser. (1988) 19:189-92), oligonucleotide-fatty acid conjugates (Grabarek et al., Anal. Biochem. (1990) 185:131-35; Staros et al., Anal. Biochem. (1986) 156:220-22), and oligonucleotide-sterol conjugates (Boujrad et al., Proc. Natl. Acad. Sci. USA (1993) 90:5728-31).

The linkage of the ISS-PN to a oligosaccharide can be formed using standard known

methods. These methods include, but are not limited to, the synthesis of oligonucleotide-oligosaccharide conjugates, wherein the oligosaccharide is a moiety of an immunoglobulin (O'Shannessy et al., J. Applied Biochem. (1985) 7:347-55).

Adjuvants and cytokines may also be genetically or chemically linked to the ISS-ODN conjugates. Examples of this type of fusion peptide are known to those skilled in the art and can also be found in Czerkinsky *et al.*, *Infect. Immun.*, 57: 1072-77 (1989); Nashar *et al.*, *Vaccine,* 11: 235-40 (1993); and Dertzbaugh and Elson, *Infect. Immun.*, 61: 48-55 (1993).

The linkage of a circular ISS-PN to an IMM can be formed in several ways. Where the circular PN is synthesized using recombinant or chemical methods, a modified nucleoside (Ruth, in *Oligonucleotides and Analogues: A Practical Approach* (1991) IRL Press). Standard linking technology can then be used to connect the circular ISS-PN to the antigen or immunostimulatory peptide (Goodchild, Bioconjugate Chem. (1990) 1: 165). Where the circular ISS-PN is isolated, or synthesized using recombinant or chemical methods, the linkage can be formed by chemically activating, or photoactivating, a reactive group (e.g. carbene, radical) that has been incorporated into the antigen or immunostimulatory peptide.

Additional methods for the attachment of peptides and other molecules to ISS-PNs can be found in C. Kessler: *Nonradioactive labeling methods for nucleic acids* in L.J. Kricka (ed.) "Nonisotopic DNA Probe Techniques," Academic Press 1992 and in Geoghegan and Stroh, *Bioconjug. Chem.*, 3:138-146, 1992.

D. *Methods and Routes for Administration of ISS-PN/IMM to a Host*

1. Drug delivery

The ISS-PN/IMM of the invention are administered to a host using any available method and route suitable for drug delivery, including *ex vivo* methods (e.g., delivery

of cells incubated or transfected with an ISS-PN/IMM) as well as systemic or localized routes. However, those of ordinary skill in the art will appreciate that methods and localized routes which direct the ISS-PN/IMM into antigen-sensitized tissue will be preferred in most circumstances to systemic routes of administration, both for immediacy of therapeutic effect and avoidance of *in vivo* degradation.

The entrance point for many exogenous antigens into a host is through the skin or mucosa. Thus, delivery methods and routes which target the skin (e.g., for cutaneous and subcutaneous conditions) or mucosa (e.g., for respiratory, ocular, lingual or genital conditions) will be especially useful. Those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, means for drug delivery into skin and mucosa. For review, however, exemplary methods and routes of drug delivery useful in the invention are briefly discussed below.

Intranasal administration means are particularly useful in addressing respiratory inflammation, particularly inflammation mediated by antigens transmitted from the nasal passages into the trachea or broncheoli. Such means include inhalation of aerosol suspensions or insufflation of the polynucleotide compositions of the invention. Nebulizer devices suitable for delivery of polynucleotide compositions to the nasal mucosa, trachea and bronchioli are well-known in the art and will therefore not be described in detail here. For general review in regard to intranasal drug delivery, those of ordinary skill in the art may wish to consult Chien, *Novel Drug Delivery Systems*, Ch. 5 (Marcel Dekker, 1992).

Dermal routes of administration, as well as subcutaneous injections, are useful in addressing allergic reactions and inflammation in the skin. Examples of means for delivering drugs to the skin are topical application of a suitable pharmaceutical preparation, transdermal transmission, injection and epidermal administration.

For transdermal transmission, absorption promoters or iontophoresis are suitable

methods. For review regarding such methods, those of ordinary skill in the art may wish to consult Chien, *supra* at Ch. 7. Iontophoretic transmission may be accomplished using commercially available "patches" which deliver their product continuously via electric pulses through unbroken skin for periods of several days or more. Use of this method allows for controlled transmission of pharmaceutical compositions in relatively great concentrations, permits infusion of combination drugs and allows for contemporaneous use of an absorption promoter.

An exemplary patch product for use in this method is the LECTRO PATCH trademarked product of General Medical Company of Los Angeles, CA. This product electronically maintains reservoir electrodes at neutral pH and can be adapted to provide dosages of differing concentrations, to dose continuously and/or to dose periodically. Preparation and use of the patch should be performed according to the manufacturer's printed instructions which accompany the LECTRO PATCH product; those instructions are incorporated herein by this reference.

Epidermal administration essentially involves mechanically or chemically irritating the outermost layer of the epidermis sufficiently to provoke an immune response to the irritant. An exemplary device for use in epidermal administration employs a multiplicity of very narrow diameter, short tynes which can be used to scratch ISS-PN/IMM coated onto the tynes into the skin. The device included in the MONO-VACC old tuberculin test manufactured by Pasteur Merieux of Lyon, France is suitable for use in epidermal administration of ISS-PN/IMM. Use of the device is according to the manufacturer's written instructions included with the device product; these instructions regarding use and administration are incorporated herein by this reference to illustrate conventional use of the device. Similar devices which may also be used in this embodiment are those which are currently used to perform allergy tests.

Opthalmic administration (e.g., for treatment of allergic conjunctivitis) involves invasive or topical application of a pharmaceutical preparation to the eye. Eye drops,

topical cremes and injectable liquids are all examples of suitable mileaus for delivering drugs to the eye.

Systemic administration involves invasive or systemically absorbed topical administration of pharamaceutical preparations. Topical applications as well as intravenous and intramuscular injections are examples of common means for systemic administration of drugs.

2. Dosing parameters

A particular advantage of the ISS-PN/IMM of the invention is their capacity to exert immunomodulatory activity even at relatively minute dosages. Although the dosage used will vary depending on the clinical goals to be achieved, a suitable dosage range is one which provides up to about 1-1000 μg of ISS-PN/IMM/ml of carrier in a single dosage. Alternatively, a target dosage of ISS-PN/IMM can be considered to be about 1-10 μM in a sample of host blood drawn within the first 24-48 hours after administration of ISS-PN/IMM. Based on current studies, ISS-PN/IMM are believed to have little or no toxicity at these dosage levels.

In this respect, it should be noted that the anti-inflammatory and immunotherapeutic activity of ISS-PN/IMM in the invention is essentially dose-dependent. Therefore, to increase ISS-PN/IMM potency by a magnitude of two, each single dose is doubled in concentration. Clinically, it may be advisable to administer the ISS-PN/IMM in a low dosage (e.g., about 1 μg/ml to about 50 μg/ml), then increase the dosage as needed to achieve the desired therapeutic goal.

In view of the teaching provided by this disclosure, those of ordinary skill in the clinical arts will be familiar with, or can readily ascertain, suitable parameters for administration of ISS-PN/IMM according to the invention.

3. ISS-PN/IMM compositions

ISS-PN/IMM will be prepared in a pharmaceutically acceptable composition for delivery to a host. Pharmaceutically acceptable carriers preferred for use with the ISS-PN/IMM of the invention may include sterile aqueous of non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/ aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. A composition of ISS-PN/IMM may also be lyophilized using means well known in the art, for subsequent reconstitution and use according to the invention.

Absorption promoters, detergents and chemical irritants (e.g., keritinolytic agents) can enhance transmission of an ISS-PN/IMM composition into a target tissue. For reference concerning general principles regarding absorption promoters and detergents which have been used with success in mucosal delivery of organic and peptide-based drugs, see Chien, *Novel Drug Delivery Systems*, Ch. 4 (Marcel Dekker, 1992).

Examples of suitable nasal absorption promoters in particular are set forth at Chien, *supra* at Ch. 5, Tables 2 and 3; milder agents are preferred. Suitable agents for use in the method of this invention for mucosal/nasal delivery are also described in Chang, *et al., Nasal Drug Delivery*, "Treatise on Controlled Drug Delivery", Ch. 9 and Table 3-4B thereof, (Marcel Dekker, 1992). Suitable agents which are known to enhance absorption of drugs through skin are described in Sloan, Use of Solubility Parameters from Regular Solution Theory to Describe Partitioning-Driven Processes, Ch. 5,

"Prodrugs: Topical and Ocular Drug Delivery" (Marcel Dekker, 1992), and at places elsewhere in the text. All of these references are incorporated herein for the sole purpose of illustrating the level of knowledge and skill in the art concerning drug delivery techniques.

A colloidal dispersion system may be used for targeted delivery of the ISS-PN/IMM to specific tissue. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. The preferred colloidal system of this invention is a liposome.

Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, *et al., Trends Biochem. Sci.*, 6:77, 1981). In addition to mammalian cells, liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells. In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the genes encoding the antisense polynucleotides at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al., Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be

used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebrosides, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes can be classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various well known linking groups can be used for joining the lipid chains to the targeting ligand (see, e.g., Yanagawa, *et al.*, *Nuc.Acids Symp.Ser.*, 19:189 (1988); Grabarek, *et al.*, *Anal.Biochem.*, 185:131 (1990); Staros, *et al.*, *Anal.Biochem.*, 156:220 (1986) and Boujrad, *et al.*, *Proc.Natl.Acad.Sci.USA*, 90:5728 (1993), the disclosures of which are incorporated

herein by reference solely to illustrate the standard level of knowledge in the art concerning conjugation of PNs to lipids). Targeted delivery of ISS-PN/IMM can also be achieved by conjugation of the ISS-PN/IMM to a the surface of viral and non-viral recombinant expression vectors, to an antigen or other ligand, to a monoclonal antibody or to any molecule which has the desired binding specificity.

Co-administration of a peptide drug with an ISS-PN/IMM according to the invention may also be achieved by incorporating the ISS-PN/IMM in *cis* or in *trans* into a recombinant expression vector (plasmid, cosmid, virus or retrovirus) which codes for any therapeutically beneficial protein deliverable by a recombinant expression vector. If incorporation of an ISS-PN/IMM into an expression vector for use in practicing the invention is desired, such incorporation may be accomplished using conventional techniques which do not require detailed explanation to one of ordinary skill in the art. For review, however, those of ordinary skill may wish to consult Ausubel, *Current Protocols in Molecular Biology*, *supra*.

D. *Screening for Active ISS-PN/IMM*

Confirmation that a particular compound has the properties of an ISS-PN/IMM useful in the invention can be obtained by evaluating whether the ISS-PN/IMM affects cytokine secretion and IgG antibody isotype production as described in Section A.I, above. Details of *in vitro* techniques useful in making such an evaluation are given in the Examples; those of ordinary skill in the art will also know of, or can readily ascertain, other methods for measuring cytokine secretion and antibody production along the parameters taught herein.

E. *Kits for Use in Practicing the Methods of the Invention*

For use in the methods described above, kits are also provided by the invention. Such kits may include any or all of the following: ISS-PN/IMM (conjugated or

unconjugated); a pharmaceutically acceptable carrier (may be pre-mixed with the ISS-PN/IMM) or suspension base for reconstituting lyophilized ISS-PN/IMM; additional medicaments; a sterile vial for each ISS-PN/IMM and additional medicament, or a single vial for mixtures thereof; device(s) for use in delivering ISS-PN/IMM to a host; assay reagents for detecting indicia that the anti-inflammatory and/or immunostimulatory effects sought have been achieved in treated animals and a suitable assay device.

Examples illustrating the practice of the invention are set forth below. The examples are for purposes of reference only and should not be construed to limit the invention, which is defined by the appended claims. All abbreviations and terms used in the examples have their expected and ordinary meaning unless otherwise specified.

EXAMPLE I

SELECTIVE INDUCTION OF A Th1 RESPONSE IN A HOST AFTER ADMINISTRATION OF AN ISS-PN/IMM

In mice, IgG 2A antibodies are serological markers for a Th1 type immune response, whereas IgG 1 antibodies are indicative of a Th2 type immune response. Th2 responses include the allergy-associated IgE antibody class; soluble protein antigens tend to stimulate relatively strong Th2 responses. In contrast, Th1 responses are induced by antigen binding to macrophages and dendritic cells.

To determine which response, if any, would be produced by mice who received ISS-PN/IMM according to the invention, eight groups of Balb/c mice were immunized with 10μg β-galactosidase protein (conjugated to avidin; Sigma, St. Louis, MO) to produce a model allergic phenotype. As set forth in the Table below, some of the mice received antigen alone, some received an antigen-ISS-PN conjugate or a conjugate using a mutant, non-stimulatory PN as a conjugate for the antigen, and others received the antigen in an unconjugated mixture with an ISS-PN. Naive mice are shown for reference:

Mouse Group	ISS-PN/IMM Treatment
1	None (β-gal antigen vaccinated)
2	DY1018-βgal conjugate (ISS-PN/IMM)
3	DY1019-βgal conjugate (PN/IMM)
4	DY1018 mixed with βgal (unconjugated)
5	βgal in adjuvant (alum)
6	plasmid DNA (ISS-ODN present but not expressible with antigen)
7	naive mice (no antigen priming)

DY1018 has the nucleotide sequence:

5'-TGACTGTG**AACGTT**CGAGATGA-3' with a phosphothioate backbone

and DY1019 has the nucleotide sequence:

5'-TGACTGTG**AAGGTT**GGAGATGA-3' with a phosphothioate backbone.

At 2 week intervals, any IgG 2a and IgG 1 to β-galactosidase present in the serum of each mouse were measured by enzyme-linked immunoabsorbent assay (using antibodies specific for the IgG 1 and IgG 2A subclasses) on microtiter plates coated with the enzyme.

As shown in FIGURE 1, only the mice who received the ISS-PN/IMM produced high titers of IgG 2A antibodies, which increased in number over a period of 8 weeks. As

shown in FIGURE 2, immunization of the mice with the antigen itself or with the PN/IMM induced production of relatively high titers of IgG 1 antibodies. The data shown in the FIGURES comprise averages of the values obtained from each group of mice.

To evaluate the effect of treatment of a host before and after a secondary antigen challenge, 3 groups of Balb/c mice were immunized with 10μg of antigen E (AgE) in alum to produce a model allergic phenotype and challenged again with the antigen, ISS-PN/IMM or mutant (nonstimulatory) PN/IMM at 5 weeks post-priming. An ELISA for IgG1 and IgG2a antibodies was performed as described 4 weeks after priming (one week before secondary antigen challenge) and again at 7 weeks (2 weeks after secondary challenge).

Again, the mice who received the ISS-PN/IMM mounted a strong Th1 type response to the antigen (IMM) as compared to the antigen-immunized and mutant PN/IMM immunized mice (FIGURE 3), while the reverse was true of a Th2 type response in the same mice (FIGURE 4).

These data indicate that a selective Th1 response is induced by administration of an ISS-PN/IMM according to the invention to both an antigen-primed (pre-antigen challenge) and an antigen-challenged host.

EXAMPLE II

SUPPRESSION OF IgE ANTIBODY RESPONSE TO ANTIGEN BY IMMUNIZATION WITH ISS-PN/IMM

To demonstrate the IgE suppression achieved through stimulation of a Th1 type cellular immune response in preference to a Th2 type cellular immune response, five

to eight week old Balb/c mice were immunized with AgE as described in the previous Example.

IgE anti-Age were detected using a solid phase radioimmunoassay (RAST) in a 96 well polyvinyl plate (a radioisotopic modification of the ELISA procedure described in Coligan, *"Current Protocols In Immunology"*, Unit 7.12.4, Vol. 1, Wiley & Sons, 1994), except that purified polyclonal goat antibodies specific for mouse ϵ chains were used in lieu of antibodies specific for human Fab. To detect anti-AgE IgE, the plates were coated with AgE (10μg/ml). The lowest IgE concentration measurable by the assay employed was 0.4ng of IgE/ml.

Measuring specifically the anti-antigen response by each group of mice, as shown in FIGURE 5, anti-AgE IgE levels in the ISS-PN/IMM immunized mice were consistently low both before and after boosting, while the protein and mutant ISS-PN/IMM injected mice developed high levels of anti-AgE after antigen challenge.

These data show that the ISS-PN/IMM immunized mice developed an antigen specific Th1 response (suppressing the Th2 IgE response) to the antigen.

EXAMPLE III

INFγ LEVELS IN MICE AFTER DELIVERY OF ISS-PN/IMM

BALB/c mice were immunized with βgal as described in Example I then sacrificed 24 hrs later. Splenocytes were harvested from each mouse.

96 well microtiter plates were coated with anti-CD3 antibody (Pharmingen, La Jolla, CA) at a concentration of 1μg/ml of saline. The anti-CD3 antibody stimulates T cells by delivering a chemical signal which mimicks the effects of binding to the T cell receptor (TCR) complex. The plates were washed and splenocytes added to each well

(4x105/well) in a medium of RPMI 1640 with 10% fetal calf serum. Supernatants were obtained at days 1, 2 and 3.

Th1 cytokine (INFγ) levels were assayed with an anti-INFγ murine antibody assay (*see*, e.g., Coligan, *"Current Protocols in Immunology"*, Unit 6.9.5., Vol. 1, Wiley & Sons, 1994). Relatively low levels of INF-γ would be expected in mice with a Th2 phenotype, while relatively high levels of INF-γ would be expected in mice with a Th1 phenotype.

As shown in FIGURE 5, levels of Th1 stimulated IFN-γ secretion were greatly increased in the ISS-PN/IMM treated mice, but substantially reduced in each other set of mice (as compared to the control), indicating development of a Th2-type phenotype in the latter mice and a Th1 phenotype in the ISS-PN/IMM treated mice.

EXAMPLE IV

BOOSTING OF CTL RESPONSES BY ISS-PN/IMM

A mixture of lymphoytes was obtained and contacted with βgal antigen alone or as part of the constructs and mixtures described in Example I. As shown in FIGURE 6, CTL production in response to ISS-PN/IMM was consistently higher than the response to antigen delivered in other forms; even **twice** as high than in animals treated with an unconjugated mixture of ISS-PN and IMM antigen.

In the experiment, the higher values for the mice treated with M-ISS-PN/IMM after antigen challenge as compared to the conventionally immunized mice is most likely owing to the antigen carrier properties of DY1019.

Thus, longer-term immunity mediated by cellular immune responses is benefitted by treatment according to the invention.

The invention claimed is:

CLAIMS

1. An immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen, conjugated to an polynucleotide that contains at least one immunostimulatory nucleotide sequence (ISS).

2. The composition of claim **1**, wherein the antigen is selected from the group consisting of proteins, glycoproteins, polysaccharides and gangliosides.

3. The composition of claim **2**, wherein the ISS comprises a nucleotide sequence selected from the group CpG, p(GC) and p(IC).

4. The composition of claim **2**, wherein the ISS comprises a CG containing oligonucleotide.

5. The composition of claim **4**, wherein the ISS further comprises a pG nucleotide sequence.

6. The composition of claim **4**, wherein the CG containing oligonucleotide has the sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

7. The composition of claim **3**, wherein the CpG, p(GC) or p(IC) containing nucleotide sequence is a palindromic double-stranded or non-palindromic single-stranded oligonucleotide.

8. The composition of claim **6**, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

9. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

10. The composition of claim 6, wherein the oligonucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

11. The composition of claim 2, wherein the polynucleotide further comprises a linear DNA sequence.

12. The composition of claim 2, wherein the polynucleotide further comprises a circular DNA sequence.

13. The composition of claim 2, wherein the polynucleotide further comprises an RNA nucleotide sequence.

14. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC.

15. The composition of claim 13, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) sequence.

16. The composition of claim 13, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

17. The composition of claim **13**, wherein the RNA nucleotide sequence comprises a sequence selected from the group consisting of AACGUU, AACGpI, AACGpC.

18. The composition of claim **2**, wherein the polynucleotide further comprises at least one modified oligonucleotide.

19. The composition of claim **11**, wherein the ISS is contained within the linear DNA sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

20. The composition of claim **11**, wherein the ISS is contained within the linear DNA sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

21. The composition of claim **12**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

22. The composition of claim **12**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

23. The composition of claim **13**, wherein the ISS is contained within the RNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

24. The composition of claim **13**, wherein the ISS is contained with the RNA nucleotide sequence, and further wherein the ISS comprises CG containing pG nucleotide sequence.

25. The composition of claim **4**, wherein the CG containing nucleotide sequence further comprises a modified oligonucleotide.

26. The composition of claim **6**, wherein the 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3' nucleotide sequence further comprises a modified oligonucleotide.

27. An immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

28. The composition of claim **27**, wherein the polynucleotide is DNA or RNA.

29. The composition of claim **27**, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

30. The composition of claim **27**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

31. The composition of claim **27**, wherein the ISS comprises a CG containing oligonucleotide.

32. The composition of claim **31**, wherein the ISS further comprises a pG nucleotide sequence.

33. The composition of claim **31**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

34. The composition of claim **31**, wherein the CG containing nucleotide sequence is a palindromic double-stranded or non-palindromic single-stranded oligonucleotide.

35. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

36. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

37. The composition of claim **33**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

38. The composition of claim **29**, wherein the polynucleotide further comprises a linear DNA nucleotide sequence.

39. The composition of claim **29**, wherein the polynucleotide further comprises a circular DNA nucleotide sequence.

40. The composition of claim **29**, wherein the polynucleotide portion further comprises an RNA nucleotide sequence.

41. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC, GACGCU, GACGCpI, GACGCpC, GACGUU, GACGpI, GACGpC, GACGUC, GACGpI, GACGpC.

42. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a double-stranded poly(I•C) nucleotide sequence.

43. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC, AGCGUC, AGCGpI, AGCGpC.

44. The composition of claim **40**, wherein the RNA nucleotide sequence comprises a nucleotide sequence selected from the group consisting of AACGUU, AACGpI, AACGpC.

45. The composition of claim **29**, wherein the polynucleotide portion further comprises at least one modified oligonucleotide.

46. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

47. The composition of claim **38**, wherein the ISS is contained within the linear DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

48. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

49. The composition of claim **39**, wherein the ISS is contained within the circular DNA nucleotide sequence, and further wherein the ISS comprises a CG containing pG nucleotide sequence.

50. The composition of claim **40**, wherein the ISS is contained within the RNA nucleotide sequence, and further wherein the ISS comprises a Purine, Purine, CG, Pyrimidine, Pyrimidine nucleotide sequence.

51. The composition of claim **40**, wherein the ISS is contained with the RNA nucleotide sequence, and further wherein the ISS comprises CG containing pG nucleotide sequence.

52. The composition of claim **31**, wherein the CG containing nucleotide sequence further comprises a modified oligonucleotide.

53. The composition of claim **33**, wherein the 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3' nucleotide sequence further comprises a modified oligonucleotide.

54. A method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory molecule, which molecule comprises an antigen, conjugated to an polynucleotide that contains at least one ISS.

55. The method of claim **54**, wherein the route of administration is a dermal route.

56. The method of claim **54**, wherein the route of administration is low-frequency ultrasonic delivery.

57. The method of claim **54**, wherein the antigen is selected from the group consisting of proteins, glycoproteins, polysaccharides and gangliosides.

58. The method of claim **57**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

59. The method of claim **57**, wherein the ISS comprises a CG containing oligonucleotide.

60. The method of claim **59**, wherein the ISS further comprises a pG nucleotide sequence.

61. The method of claim **59**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

62. The method of claim **59**, wherein the CG containing nucleotide sequence is a palindromic or non-palindromic oligonucleotide nucleotide sequence.

63. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

64. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

65. The method of claim **59**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

66. The method of claim **54**, wherein the immune response modulation comprises the induction of a Th1 response.

67. The method of claim **66**, wherein the antigen molecule is selected from the group consisting of proteins, glycoproteins and polysaccharides.

68. The method of claim **67**, wherein the ISS comprises a DNA or RNA nucleotide sequence selected from the group CG, p(GC) and p(IC).

69. The method of claim **67**, wherein the ISS comprises a CG containing oligonucleotide.

70. The method of claim **69**, wherein the ISS further comprises a pG nucleotide sequence.

71. The method of claim **69**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

72. The method of claim **69**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

73. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

74. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

75. The method of claim **69**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

76. A method of modulating an immune response comprising the administration of an immunomodulatory composition comprising an immunomodulatory molecule, which molecule is comprised of an antigen and an immunostimulatory peptide, conjugated to an polynucleotide that contains at least one ISS.

77. The method of claim **76**, wherein the route of administration is a dermal route.

78. The method of claim **76**, wherein the route of administration is low-frequency ultrasonic delivery.

79. The method of claim **76**, wherein the immunostimulatory peptide is selected from the group consisting of co-stimulatory molecules, cytokines, chemokines, targeting protein ligands, and trans-activating factors.

80. The method of claim **79**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

81. The method of claim **79**, wherein the ISS comprises a CG containing oligonucleotide.

82. The method of claim **81**, wherein the ISS further comprises a pG nucleotide sequence.

83. The method of claim **81**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

84. The method of claim **81**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

85. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

86. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

87. The method of claim **81**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

88. The method of claim **76**, wherein the immune response modulation comprises the induction of a Th1 response.

89. The method of claim **88**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

90. The method of claim **89**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

91. The method of claim **89**, wherein the ISS comprises a CG containing oligonucleotide.

92. The method of claim **91**, wherein the ISS further comprises a pG nucleotide sequence.

93. The method of claim **91**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

94. The method of claim **91**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

95. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

96. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

97. The method of claim **91**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

98. A method for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising administering a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen, to a mammalian host.

99. The method of claim **98** wherein the polynucleotide includes at least one ISS.

100. The method of claim **98** wherein the polynucleotide is free of ISS.

101. The method of claim **98**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

102. The method of claim **98**, wherein the ISS comprises a nucleotide sequence selected from the group CG, p(GC) and p(IC).

103. The method of claim **98**, wherein the ISS comprises a CG containing oligonucleotide.

104. The method of claim **103**, wherein the ISS further comprises a pG nucleotide sequence.

105. The method of claim **103**, wherein the CG containing nucleotide sequence is the nucleotide sequence 5'-Purine, Purine, CG, Pyrimidine, Pyrimidine-3'.

106. The method of claim **103**, wherein the CG containing nucleotide sequence is a double-stranded palindromic or single-stranded non-palindromic oligonucleotide nucleotide sequence.

107. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, AGCGTC, GACGTC, GGCGTC, AACGCC, AGCGCC, GACGCC, GGCGCC, AACGCT, AGCGCT, GACGCT, and GGCGCT.

108. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, GACGTT, GGCGTT, AACGTC, and AGCGTC.

109. The method of claim **102**, wherein the nucleotide sequence is selected from the group consisting of AACGTT, AGCGTT, and GACGTT.

110. The method of claim **98** wherein the polynucleotide comprises a GpG oligonucleotide.

111. The method of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

112. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, and AGGGTC.

113. The composition of claim **110**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, and GAGGTT.

114. A composition for introducing a soluble antigen into the Class I MHC processing pathway of the mammalian immune system to elicit a CTL response to the antigen comprising a polynucleotide conjugated to an immunomodulatory molecule, which molecule comprises the antigen.

115. The composition of claim **114**, wherein the antigen is selected from the group consisting of proteins, glycoproteins and polysaccharides.

116. The composition of claim **114** wherein the polynucleotide comprises a GpG oligonucleotide.

117. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, AGGGTC, GAGGTC, GGGGTC, AAGGCC, AGGGCC, GAGGCC, GGGGCC, AAGGCT, AGGGCT, GAGGCT, and GGGGCT.

118. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, GAGGTT, GGGGTT, AAGGTC, and AGGGTC.

119. The composition of claim **116**, wherein the nucleotide sequence is selected from the group consisting of AAGGTT, AGGGTT, and GAGGTT.

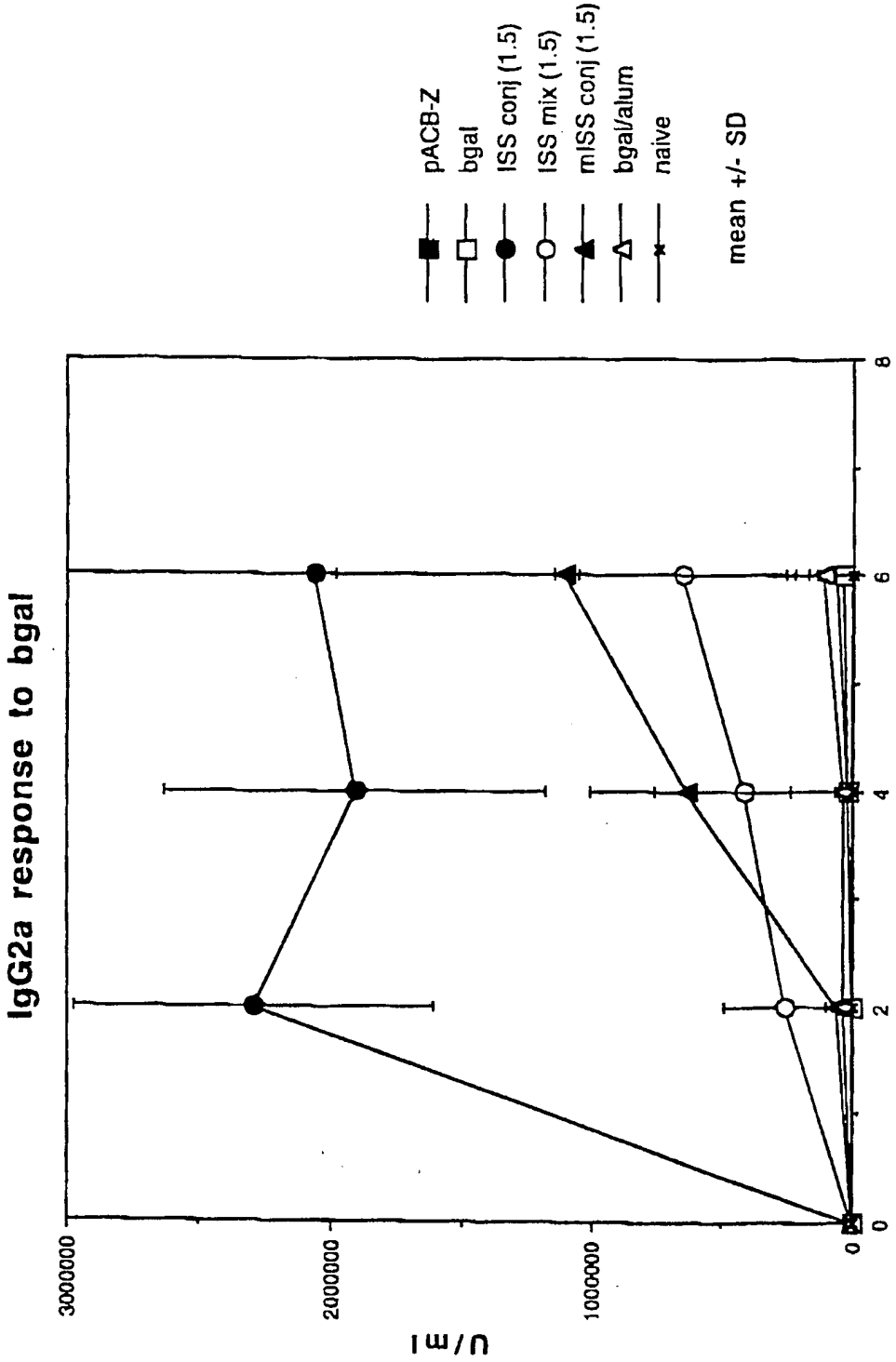
IgG2a response to bgal
U/ml
3000000
2000000
1000000
0
0
2
4
6
8
weeks
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD

FIGURE 1

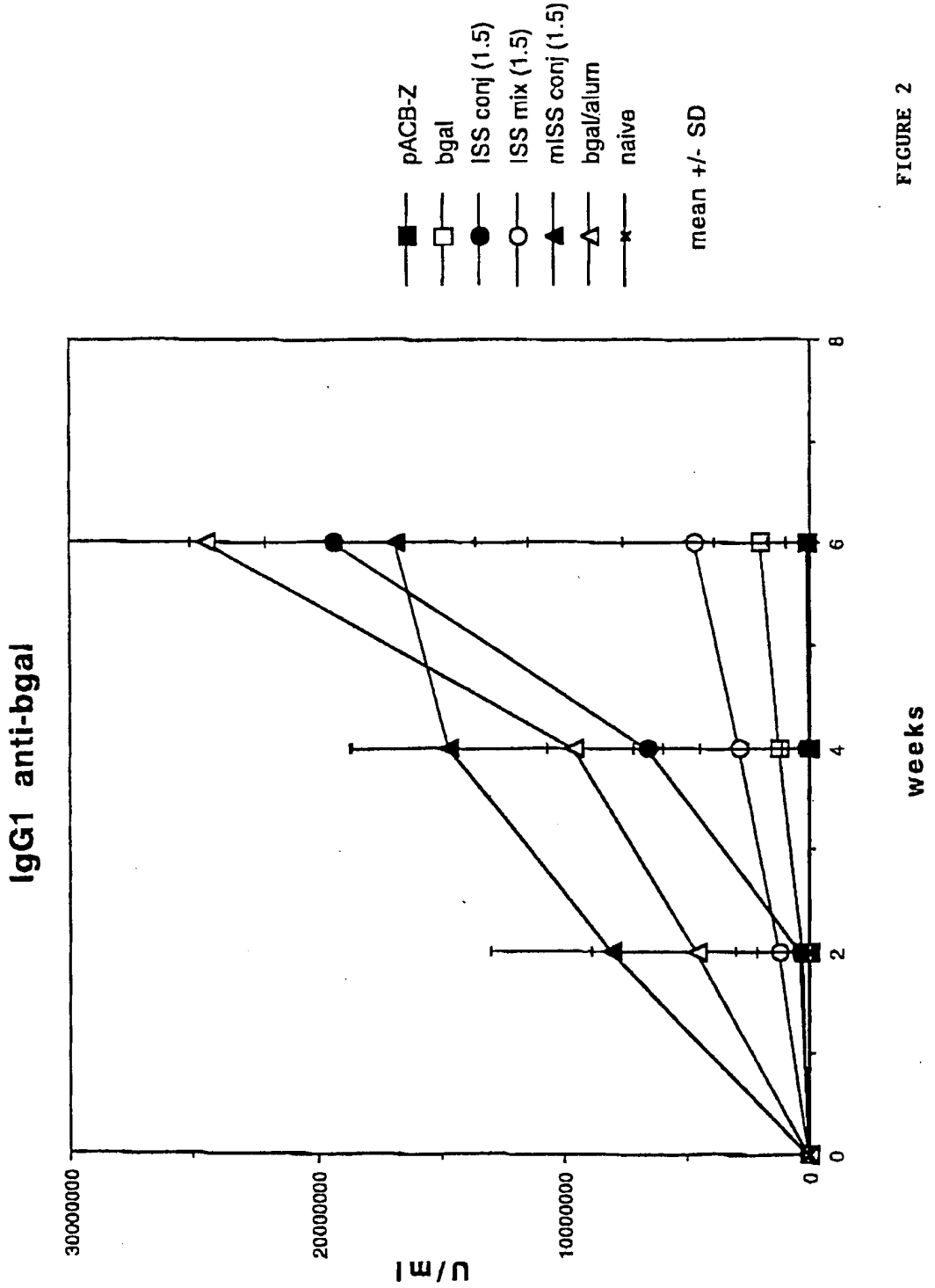
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD
IgG1 anti-bgal
U/ml
30000000
20000000
10000000
0
0
2
4
6
8
weeks

FIGURE 2

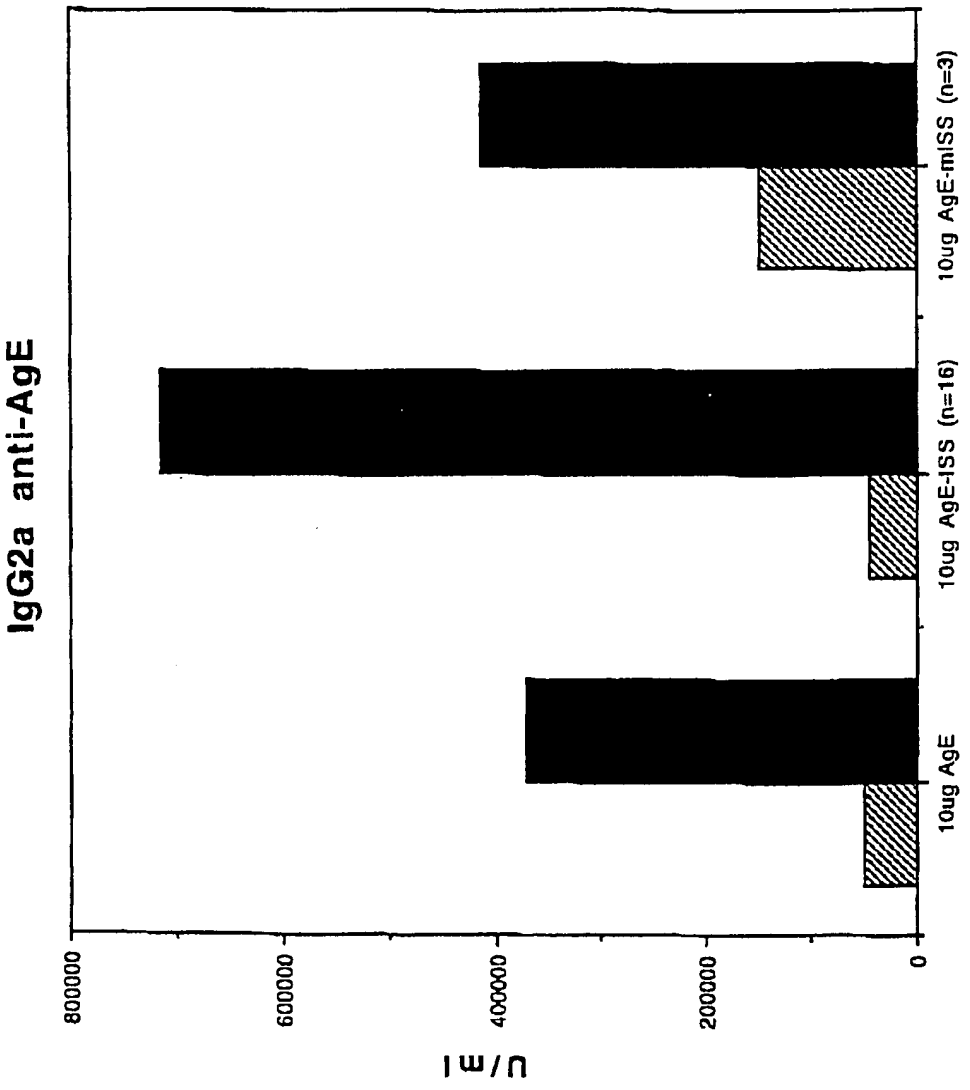
IgG2a anti-AgE
wk 4
wk 7
U/ml
800000
600000
400000
200000
0
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 3

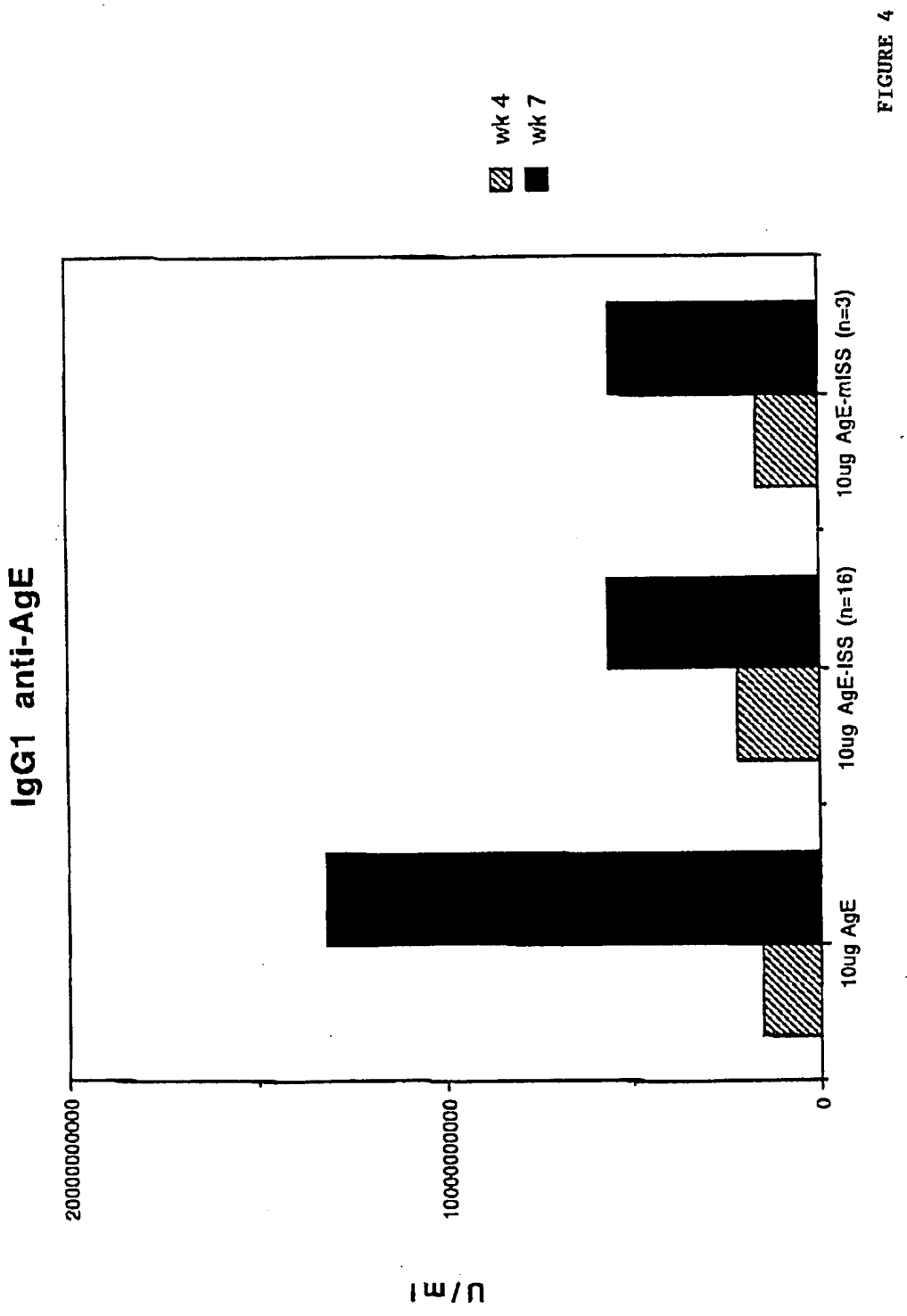
wk 4
wk 7
IgG1 anti-AgE
U/ml
20000000000
10000000000
0
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 4

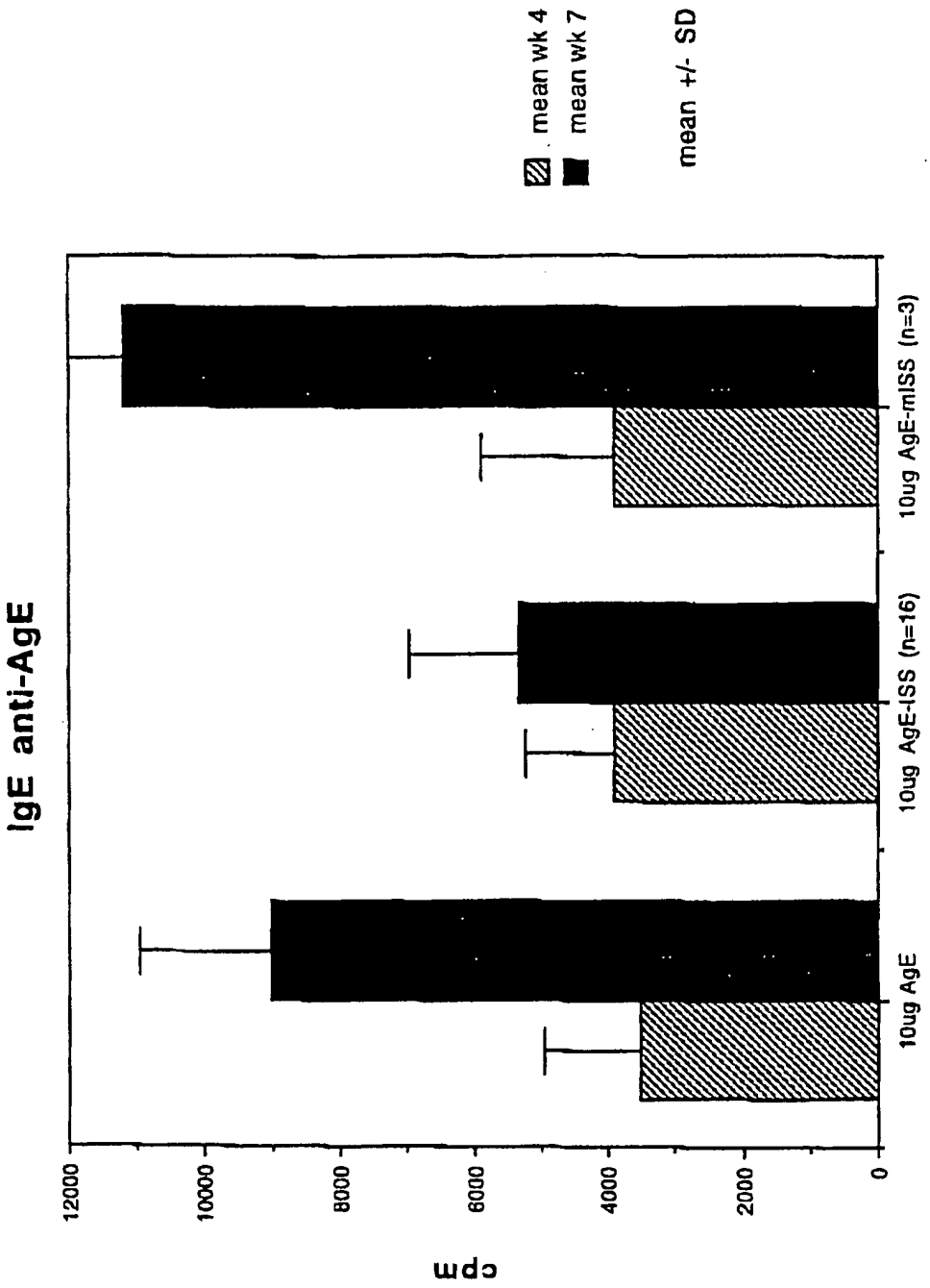
mean wk 4
mean wk 7
mean +/- SD
IgE anti-AgE
cpm
12000
10000
8000
6000
4000
2000
0
10ug AgE
10ug AgE-ISS (n=16)
10ug AgE-mISS (n=3)

FIGURE 5

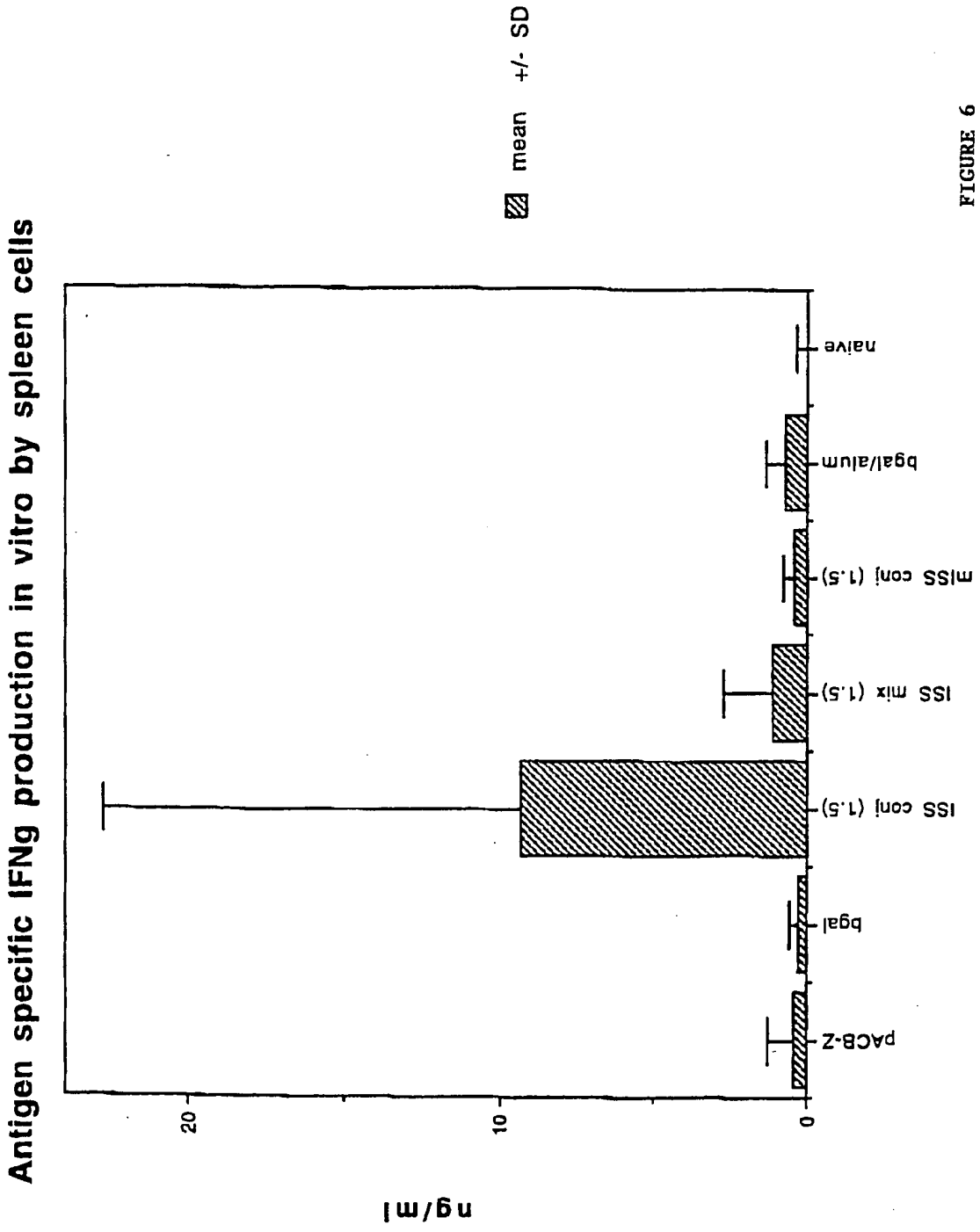
Antigen specific IFNg production in vitro by spleen cells
mean +/- SD
ng/ml
0
10
20
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive

FIGURE 6

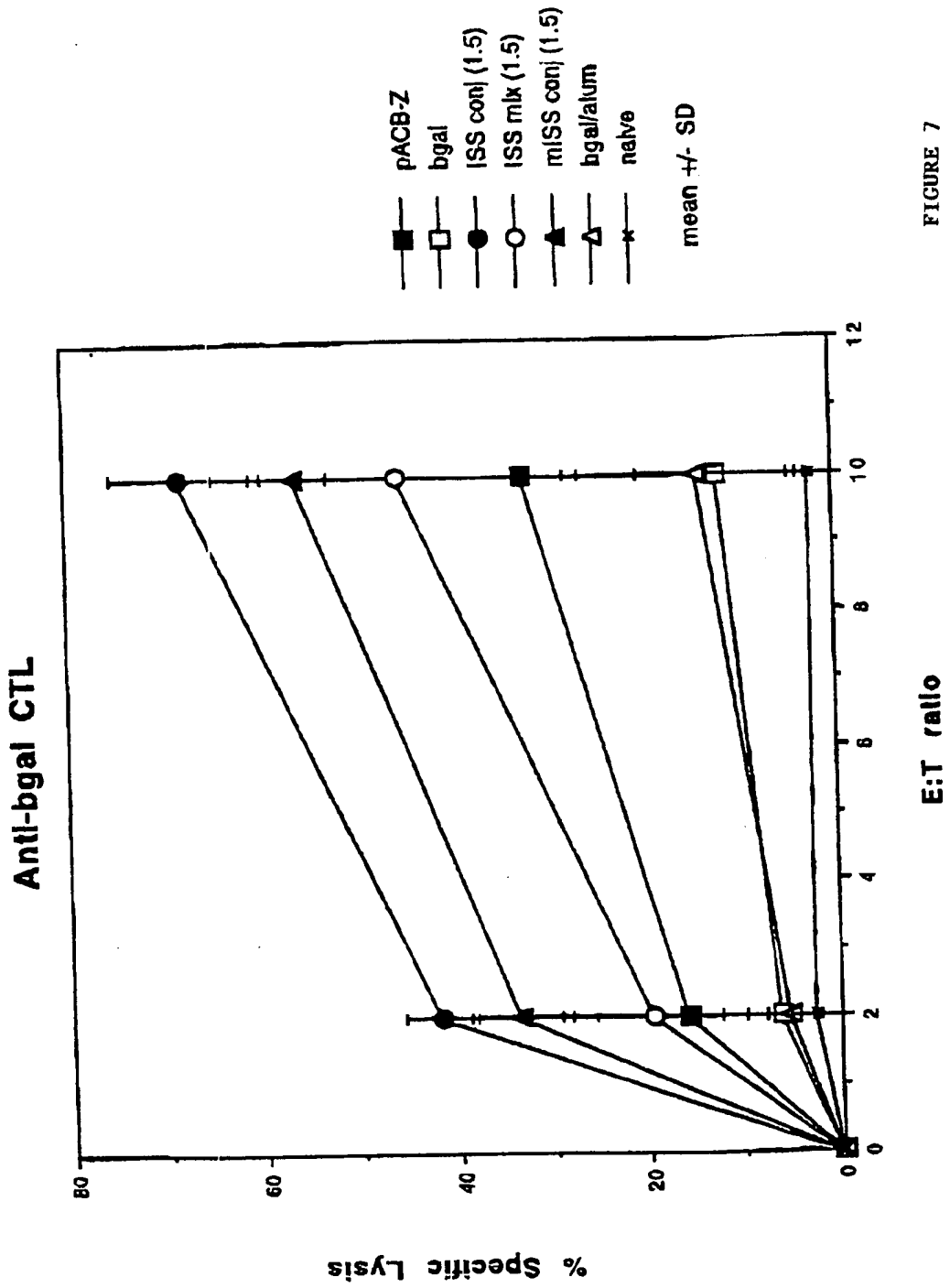
Anti-bgal CTL
% Specific Lysis
E:T ratio
pACB-Z
bgal
ISS conj (1.5)
ISS mix (1.5)
mISS conj (1.5)
bgal/alum
naive
mean +/- SD
0
20
40
60
80
0
2
4
6
8
10
12

FIGURE 7

INTERNATIONAL SEARCH REPORT

Intern ıal Application No
PCT/US 97/19004

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/00 A61K39/385 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 02555 A (UNIV IOWA RES FOUND) 1 February 1996 see page 7, line 5 - page 8, line 6 see page 11, line 10 - line 20; table 1 see page 21, line 18 - line 21	1,54
Y	---	2-53, 55-119
Y	IVAN M. ROIT: "ENCYCLOPEDIA OF IMMUNOLOGY" 1992 , ACADEMIC PRESS , LONDON XP002058362 see page 28 - page 30 see page 30, left column, first paragraph	2-53, 55-119

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
10 March 1998	25. 03. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fernandez y Branas, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern: ıal Application No
PCT/US 97/19004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAZ E. ET AL: "Potential role of immunostimulatory DNA sequences (ISS) in genetic immunization and autoimmunity" ARTHRITIS & RHEUMATISM, vol. 39, no. 9, September 1996, page 615 XP002058356 see the whole document ---	1-119
A	SATO Y. ET AL: "Immunostimulatory DNA sequences necessary for effective intradermal gene immunization" SCIENCE, vol. 273, July 1996, LANCASTER, PA US, XP002058357 see the whole document ---	1-119
A	ARTHUR M. KRIEG ET AL: "CpG motifs in bacterial DNA trigger direct B-cell activation" NATURE, vol. 374, 1995, LONDON GB, pages 546-549, XP002058358 see the whole document ---	1-119
A	BALLAS, Z.K. ET AL: "Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA" JOURNAL OF IMMUNOLOGY, vol. 157, September 1996, BALTIMORE US, pages 1840-1845, XP002058359 see the whole document ---	1-119
A	RAZ E. ET AL: "Preferential induction of a Th1 immune response and inhibition of specific IgE antibody formation by plasmid DNA immunization" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, May 1996, WASHINGTON US, pages 5141-5145, XP002058360 see the whole document ---	1-119
A	BRANDA R.F. ET AL: "Amplification of antibody production by phosphorothioate oligodeoxynucleotides" THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE, vol. 128, no. 3, September 1996, pages 329-338, XP002058361 see the whole document ---	1-119
A	WO 95 26204 A (ISIS PHARMACEUTICALS INC) 5 October 1995 see the whole document ---	1-119

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/19004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 906 092 A (HILLEMAN MAURICE R ET AL) 16 September 1975 see the whole document ---	1-119
A	US 3 725 545 A (MAES R) 3 April 1973 see the whole document ---	1-119
A	GB 1 234 718 A (MERCK) 9 June 1971 see the whole document -----	1-119

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/19004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 112-113 (partially)

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claims 112-113 refer to the compositions of claim 110; however, claim 110 is a method claim. This is obscure. Hence, claims 112-113 have been understood as method claims.

Remark : Although claims 54-113
are directed to a method of treatment of
the human/animal body , the search has been carried out and based on the alleged effects of the composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ial Application No
PCT/US 97/19004

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CELLULAR IMMUNOLOGY 190, 77–82 (1998)
ARTICLE NO. CI981400

RAPID COMMUNICATION

Immunostimulatory DNA Is a Potent Mucosal Adjuvant[1]

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Most proteins delivered to mucosal surfaces fail to induce mucosal or systemic immune responses. We demonstrate that a single intranasal (i.n.) coadministration of a model antigen (β-galactosidase, β-gal) with immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) induces a mucosal IgA response equivalent to that induced by i.n. codelivery of β-gal with cholera toxin (CT). Furthermore, i.n. and intradermal (i.d.) delivery of the β-gal/ISS-ODN mix stimulates equivalent Th_1-biased systemic immune responses with high-level cytotoxic T lymphocyte (CTL) activity. In contrast, i.n. immunization with β-gal and CT results in a Th_2-biased systemic immune response with poor CTL activity. Our data show that i.n. delivery of ISS-ODN provides effective adjuvant activity for the induction of both mucosal and systemic Th_1-biased immune responses. This immunization approach deserves consideration in the development of vaccines against mucosal pathogens. © 1998 Academic Press

Key Words: **immunostimulatory sequence DNA; mucosal adjuvant; IgA; vaccination.**

INTRODUCTION

The respiratory, gastrointestinal, vaginal, and rectal mucosa are sites where the majority of infectious agents are first encountered (1, 2). These surfaces are protected by secreted IgA (1–3). With intracellular pathogens, a CTL[3] response is important for elimination of the infectious agent (2, 4). Natural infection often induces these protective immune responses (1, 2). In contrast, delivery of monomeric protein antigens via mucosal routes generally does not stimulate any immune response, and delivery by systemic routes (i.e., i.d. and intramuscular, i.m.) leads to serum antibody production but mucosal IgA and CTL activity are not induced (1, 2). To produce a more comprehensive immune response to protein antigens, the use of adjuvants, including the mucosal adjuvant CT, has been explored.

Immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) have previously been shown to provide effective adjuvant activity for the induction of systemic Th_1-biased immunity toward protein antigens coadministered via i.d. and i.m. routes (5–9). The immune response includes the induction of a Th_1 cytokine profile (IFN-γ but not IL-4), the production of high IgG2a and low IgG1 titers, and a CTL response (5–9). In this article we expand upon previous observations regarding the potent Th_1-biased adjuvant effect of ISS-ODN and demonstrate that, in addition, it is as good a mucosal adjuvant as CT. We show that i.n. administration of β-gal with either ISS-ODN or CT leads to equivalent mucosal IgA responses. In addition, i.n. and i.d. codelivery of β-gal with ISS-ODN induces equivalent Th_1-biased serum IgG subclass, splenic cytokine, and CTL responses, while i.n. β-gal/CT codelivery leads to a Th_2-biased systemic immune response. In considering the potential application of ISS-ODN as a vaccine adjuvant against mucosal pathogens, our data suggest that i.n. antigen/ISS-ODN delivery is superior to i.d. delivery for the induction of protective immunity.

MATERIALS AND METHODS

Immunization reagents. β-Gal and CT (Sigma, St. Louis, MO), ISS-ODN, and mutated phosphorothioate oligodeoxynucleotide (M-ODN) (Trilink Biotechnologies, San Diego, CA) were used to immunize mice. The ISS-ODN used in these studies has the following se-

[1] This work was supported in part by NIH Grants AI01490 and AI40682 and by Dynavax Technologies Corporation.

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[3] Abbreviations used: i.n., intranasal; i.d., intradermal; i.g., intragastric; β-gal, β-galactosidase; ISS-ODN, immunostimulatory sequence oligodeoxynucleotide; M-ODN, mutated oligodeoxynucleotide; CT, cholera toxin; CTL, cytotoxic T lymphocyte; BALF, bronchoalveolar lavage fluid.

0008-8749/98 $25.00

quence 5′-TGACTGTGAACGTTCGAGATGA-3′. The M-ODN has the sequence 5′-TGACTGTGAACCTTAGAGATGA-3′.

Immunization protocols. Female BALB/c mice 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and used in all experiments. Intranasal immunizations were performed with β-gal (50 μg) alone or mixed with 50 μg of ISS-ODN or M-ODN, or with CT (10 μg) in 30 μl of saline. Mice were anesthetized with Metofane (Mallinckrodt Veterinary Inc., Mundelein, IL) and 15 μl was delivered to each nare. Alternatively, mice received β-gal (50 μg) plus ISS-ODN (50 μg) in 50 μl of saline injected i.d. into the base of the tail, or β-gal (200 μg) plus ISS-ODN (50 μg) administered intragastrically (i.g.) by blunted needle in 400 μl of 0.2 M Na bicarbonate. Mice were fasted for 4 h before i.g. immunization.

Bronchoalveolar lavage and fecal IgA extraction. Bronchoalveolar lavage fluid (BALF) was obtained by cannulation of the trachea of sacrificed mice during week 7. The lungs were then flushed with 0.8 ml of PBS. The return was spun to remove cellular debris, and frozen at −70°C until IgA assay. Feces were collected at 2-week intervals and IgA was extracted according to a previously published protocol (10). Briefly, three to six pieces of freshly voided feces were collected and subsequently dried in a Speed Vac Concentrator. After feces were dried, net dry weights were recorded, and the material was resuspended in PBS with 5% nonfat dry milk and protease inhibitors at a ratio of 20 μl/mg of feces to standardize for variability in the amount of fecal material collected (10). The solid matter was resuspended by vortexing for 2 h followed by centrification at 16,000*g* for 10 min to separate residual solids from supernatant. Supernatants were then frozen at −70°C until IgA assay.

Immunologic assays. Serum, BALF, and fecal extraction fluid were used in ELISA assays for antigen-specific immunoglobulin as described previously (8, 9). Results are expressed in units/milliliter based on pooled high titer anti-β-gal standards obtained from mice receiving multiple immunizations. The undiluted fecal IgA and serum IgG standards were given arbitrary concentrations of 2000 and 400,000 U/ml respectively. Samples were compared to the standard curve on each plate using the DeltaSOFT II v. 3.66 program (Biometallics, Princeton, NJ). Mouse spleens were harvested at week 7 for CTL and cytokine assays. For CTL assays, 7×10^6 splenocytes from immunized mice were incubated with 6×10^6 mitomycin C-treated naive splenocytes in the presence of recombinant human IL-2 and class I $H2^d$-restricted β-gal nanopeptide (T-P-H-P-A-R-I-G-L) as previously described (9). After 5 days, restimulated cells were harvested and specific lysis of target cells measured (9). Splenocyte cytokine profiles were conducted by incubation of 5×10^5 splenocytes in 96-well plates in a final volume of 200 μl of supplemented RPMI 1640 with β-gal added at 10 μg/ml, at 37°C/5% CO_2 as previously described (8, 9). Culture supernatants were harvested at 72 h and analyzed by ELISA. A standard curve was generated using known amounts of recombinant IFN-γ (PharMingen, San Diego, CA) and IL-4 (Genzyme, Cambridge, MA). Each culture supernatant was compared to the standard curve on the plate using the DeltaSOFT II v. 3.66

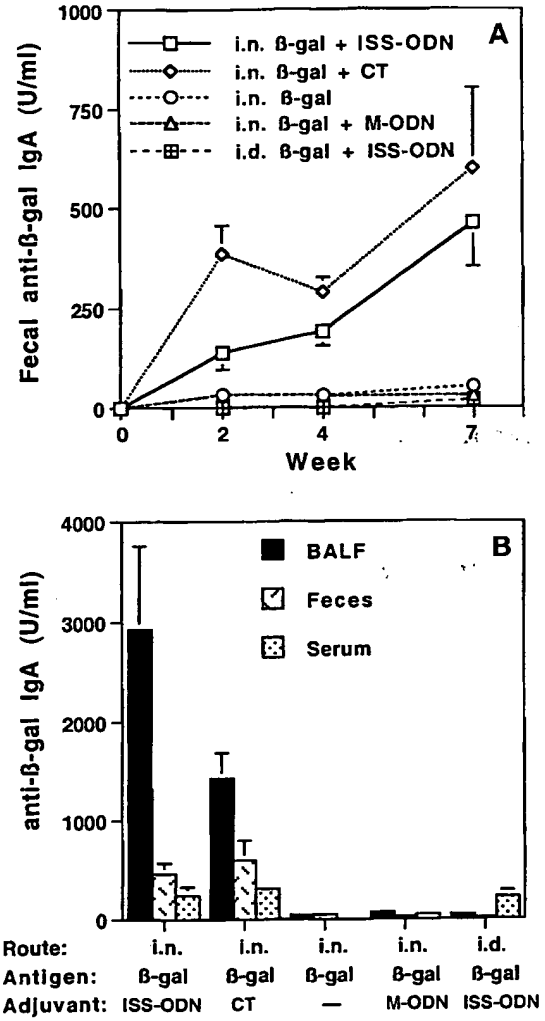

FIG. 1. IgA responses. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Results were obtained by ELISA and represent mean values for 4 mice per group. Error bars reflect the standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Fecal IgA. Feces were collected at 2, 4, and 7 weeks and IgA extracted as described under Materials and Methods. There was no significant difference in anti-β-gal IgA levels between the i.n. β-gal/ISS-ODN and i.n. β-gal/CT vaccinated groups except at 2 weeks ($p = 0.03$). (B) BALF and serum IgA. BALF and serum were obtained at sacrifice during week 7 and compared to week 7 fecal IgA. There was no significant difference in the BALF anti-β-gal IgA levels between i.n. β-gal/ISS-ODN and i.n. β-gal/CT immunized groups.

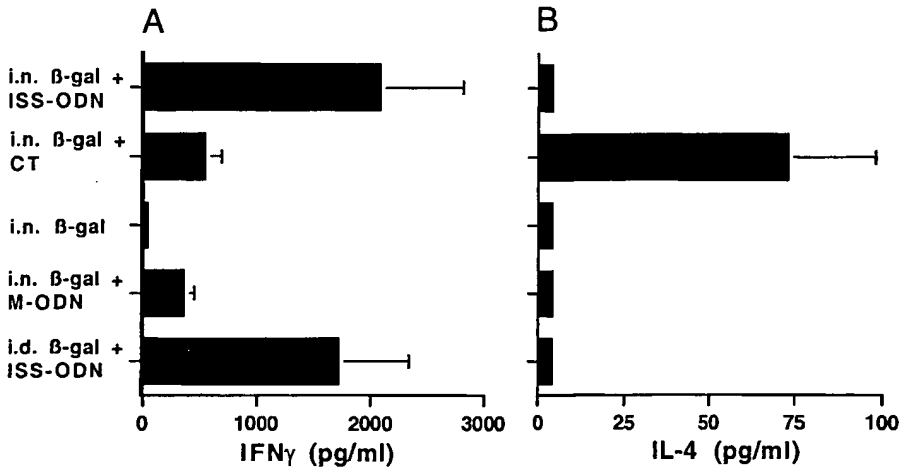

FIG. 2. Antigen-induced cytokine profiles. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Splenocytes were harvested from sacrificed mice during week 7 and cultured in media with or without β-gal (10 μg/ml), and 72-h supernatants were assayed by ELISA. Splenocytes cultured without β-gal produced no detectable IFN-γ or IL-4 (data not shown). Results represent the mean for 4 mice in each group and similar results were obtained in 2 other independent experiments. Error bars reflect standard errors of the means. (A) IFN-γ levels. IFN-γ levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups ($P = 0.05$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT vaccinated mice). (B) IL-4 levels. IL-4 levels above background were detected only in mice immunized with i.n. β-gal/CT ($P = 0.04$ verses background).

program. Statistical analysis of results was conducted using Statview computer software (Abacus Concepts, Grand Rapids, MI). A two-tailed Student t test was used to establish p values, and those ≤ 0.05 were considered significant.

RESULTS AND DISCUSSION

ISS-ODN is an effective mucosal adjuvant. Cholera toxin is the most potent known mucosal adjuvant (2). Therefore, the mucosal IgA response of mice immunized with i.n. β-gal/ISS-ODN and β-gal/CT were compared. As can be seen in Fig. 1, at 7 weeks post β-gal/ISS-ODN and β-gal/CT vaccination the mean anti-β-gal IgA levels were 462 and 599 U/ml in fecal material and 2935 and 1432 U/ml in BALF, respectively. Differences in mucosal IgA levels between i.n. β-gal/ISS-ODN-immunized and i.n. β-gal/CT-immunized mice were not statistically significant. To establish that a mucosal adjuvant was needed for the induction of mucosal IgA, we vaccinated mice i.n. with β-gal alone or with M-ODN. However, i.n. immunization without mucosal adjuvant resulted in no detectable IgA. We next evaluated whether contact with the respiratory mucosa was required for ISS-ODN to have mucosal adjuvant activity. Mice were therefore vaccinated with β-gal and ISS-ODN via i.d. and i.g. routes. These routes of immunization did not lead to measurable IgA in mucosal secretions (data for i.g. immunization not shown). To establish whether the IgA detected in fecal material and BALF of vaccinated mice was actively secreted by mucosal tissue or passively diffused from serum, anti-β-gal IgA levels in serum, fecal material, and BALF were compared. It should be noted that initial acquisition of BALF and fecal samples required an unmeasurable dilution of the IgA content of the material which does not occur when obtaining serum. Despite this fact, i.n. β-gal/ISS-ODN-immunized and i.n. β-gal/CT-immunized mice demonstrated higher levels of anti-β-gal IgA in feces and BALF then in serum, strongly suggesting that active secretion of anti-β-gal IgA from mucosal surfaces occurred in these mice (Fig. 1B).

These results demonstrate that ISS-ODN and CT have equivalent mucosal adjuvant activity with a test antigen that has no capacity to induce mucosal IgA production when delivered alone. In addition, we show that i.d. delivery of β-gal with ISS-ODN does not lead to a mucosal IgA response. Taken together these findings show that ISS-ODN is an excellent adjuvant for the induction of mucosal immunity when codelivered with antigen via the nose.

Immunization with β-gal and ISS-ODN by the i.n. route induces a vigorous Th_1-biased systemic immune response. We next evaluated the magnitude and phenotype of the systemic immune response induced by i.n. β-gal/ISS-ODN immunization. Splenocytes were harvested from mice 7 weeks after vaccination and incubated with β-gal. Culture supernatants were assayed for the production of IFN-γ and IL-4, cytokines classically associated with Th_1 and Th_2 immunity respectively (11, 12) (Fig. 2). Splenocytes from mice immunized with β-gal and ISS-ODN via the i.n. and i.d. routes produced a mean of 2084 and 1720 pg/ml of

IFN-γ, respectively (p value not significant), but no detectable IL-4. In contrast, i.n. vaccination with β-gal and CT led to splenocyte production of a mean of 542 pg/ml of IFN-γ and 73 pg/ml of IL-4 ($p = 0.05$ for both IFN-γ and IL-4 when compared to i.n. β-gal/ISS-ODN vaccination). Intranasal immunization with β-gal alone or with M-ODN led to much lower or undetectable cytokine production from splenocytes.

IFN-γ is a switch factor for IgG2a production, while IL-4 is a switch factor for IgG1 (11, 12). Given the splenic cytokine profiles, it would therefore be predicted that i.n. β-gal/ISS-ODN coadministration would lead to higher IgG2a and lower IgG1 levels then i.n. β-gal/CT codelivery. Indeed, we found that i.n. and id β-gal/ISS-ODN-immunized mice produced equivalent Th_1-biased serum antibody responses, whereas i.n. β-gal/CT vaccination led to a Th_2-biased IgG subclass profile.

At 7 weeks post-i.n. and i.d. β-gal/ISS-ODN immunization mean serum anti-β-gal IgG2a levels were 306,144 and 362,850 U/ml, and anti-β-gal IgG1 levels were 5971 and 3676 U/ml, respectively (Fig. 3). These differences were not statistically significant. In contrast, i.n. vaccination with β-gal and CT induced mean serum IgG2a and IgG1 levels of 94,518 and 36,471 U/ml ($p = 0.005$ for IgG2a and $P = 0.004$ for IgG1 compared to i.n. β-gal/ISS-ODN immunization). Again, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable IgG responses.

Cumulatively, these observations demonstrate that i.n. and i.d. delivery of antigen with ISS-ODN lead to equivalent Th_1-biased cytokine and antibody profiles, whereas i.n. β-gal/CT coadministration leads to a Th_2-biased systemic immune response. Considered in conjunction with the IgA data previously presented, we further demonstrate that production of mucosal IgA can occur in the context of both Th_1- and Th_2-biased systemic immune responses.

Codelivery of β-gal plus ISS-ODN by the i.n. route induces a strong splenic CTL response. Although development of antigen-specific CTL activity is associated with Th_1-biased immunity, not all Th_1-biased immune responses include the development of cytotoxic T cells (2, 4). Therefore, we next evaluated the ability of i.n. codelivery of β-gal and ISS-ODN to induce a CTL response. As demonstrated in Fig. 4, mice immunized with β-gal and ISS-ODN by either the i.n. or i.d. route displayed vigorous splenic CTL activity. At an E:T ratio of 5:1, i.n. and i.d. codelivery of β-gal/ISS-ODN led to 52 and 39% specific lysis of target cells, respectively. The difference was not statistically significant. However, i.n. β-gal/CT vaccination resulted in only 3% specific lysis at the same E:T ratio ($p = 0.005$ compared to vaccination with i.n. β-gal/ISS-ODN). Likewise, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable CTL responses. These results

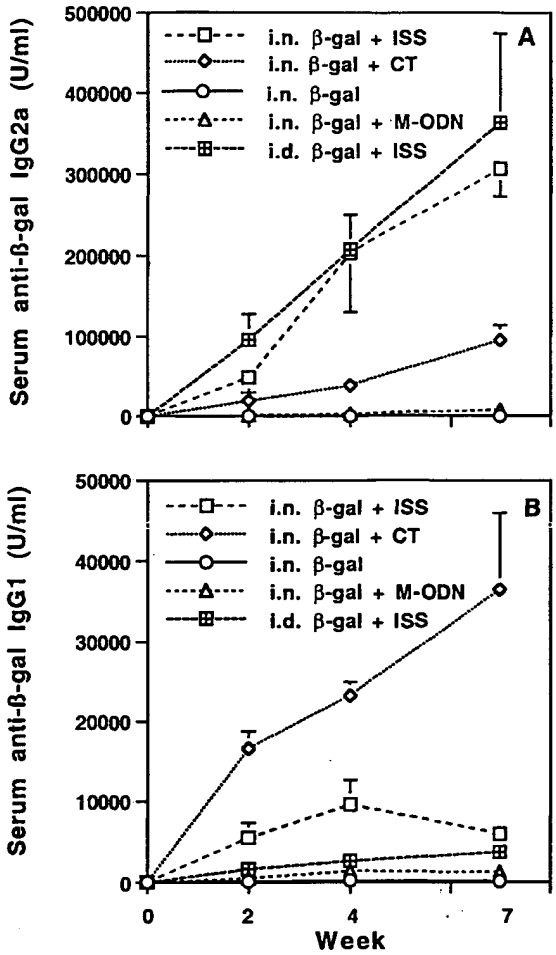

FIG. 3. IgG subclass profiles. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Serum was collected at 2, 4, and 7 weeks from immunized mice and assayed by ELISA. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Serum IgG2a. Serum IgG2a levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups at 7 weeks ($p = 0.005$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice). (B) Serum IgG1. Serum IgG1 levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically lower than in i.n. β-gal/CT-immunized mice at all time points ($p = 0.003$, $p = 0.02$, and $p = 0.02$ for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice at 2, 4, and 7 weeks, respectively).

show that while i.n. and i.d. β-gal/ISS-ODN coimmunization leads to equivalent and robust CTL responses, i.n. β-gal/CT coadministration leads to a poor CTL response. In addition, the CTL assay results further demonstrate the dichotomy between the Th_1- and Th_2-biased systemic immune responses seen when β-gal is codelivered i.n. with ISS-ODN or with CT, respectively.

In summary, our findings demonstrate that i.n. delivery of antigen with either ISS-ODN or CT leads to an equivalent and vigorous mucosal IgA response, whereas i.d. codelivery of antigen with ISS-ODN does

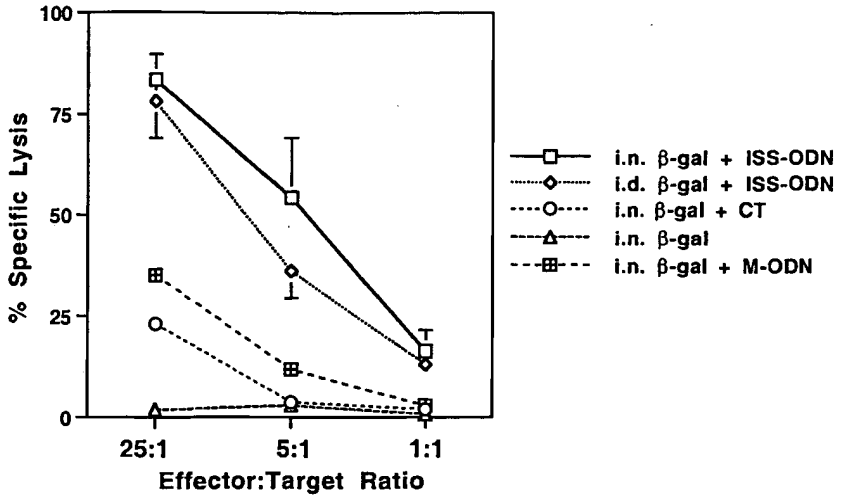

FIG. 4. CTL responses. Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Splenocytes were harvested from mice at week 7 and CTL responses were determined as outlined under Materials and Methods. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. CTL responses were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice at all E:T ratios, but statistically higher than in i.n. β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1 ($p = 0.005$ and $p = 0.05$ for i.n. β-gal/ISS-ODN versus in β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1, respectively).

not. However, i.d. and i.n. vaccination with β-gal and ISS-ODN induce equivalent systemic Th_1-biased immune responses characterized by high levels of antigen-specific IFN-γ but no IL-4 production from cultured splenocytes, high IgG2a and low IgG1 serum concentrations, and vigorous CTL responses. In contrast, i.n. codelivery of β-gal with CT leads to a Th_2-biased systemic immune response characterized by low IFN-γ but substantial IL-4 production from *in vitro* antigen-stimulated splenocytes, high IgG1 and low IgG2a serum concentrations, and a poor CTL response. The observation of equivalent mucosal IgA levels in the context of Th_1-biased and Th_2-biased systemic immune responses with i.n. β-gal/ISS-ODN and β-gal/CT immunizations respectively is consistent with other published results (18). Mariarosaria and colleagues recently demonstrated that oral delivery of tetanus toxoid with CT led to mucosal IgA production in conjunction with a Th_2 systemic immune profile and that coadministration with oral IL-12 skewed the systemic immune response toward a Th_1 phenotype, whereas mucosal IgA production was unaffected (18). Taken together, these findings document that synthesis of mucosal IgA can occur in the context of both Th_1- and Th_2-biased systemic immunity.

Mucosal IgA and CTL responses are known to provide protection against a number of infectious agents (1–3). HIV is but one example (4, 13, 14). There are a number of strategies available for the development of vaccines which induce these immune parameters. However, none at present appear globally applicable (15). Live attenuated vaccines produce robust immunity including mucosal IgA and CTL responses. Unfortunately, difficulty in attenuating many pathogens and the risk of iatrogenic disease limits the use and development live attenuated vaccines (1, 2, 13, 15). On the other hand, recombinant proteins from infectious agents are generally safe but induce relatively poor immune responses, and are not active when delivered to mucosal surfaces (1, 2). However, mucosal adjuvants can improve immune responses toward coadministered protein antigens substantially (1, 2, 15). Cholera toxin is an extremely potent mucosal adjuvant, but is inherently toxic and induces a Th_2-biased immune response that includes the development of IgE and allergic sensitization toward the target antigen (16, 17). At present, such toxicity and other technical problems have kept many adjuvants from becoming available for use in humans (15). Alum is essentially the only adjuvant in clinical use today. It is relatively weak, does not work with a number of antigens, does not induce CTL activity, and, because it must be delivered systemically, does not induce mucosal IgA (15). A safe and effective mucosal adjuvant would be of great value in the development of better vaccines. ISS-ODN is a potent adjuvant which works with a wide range of protein antigens, and generally induces a Th_1-biased immune response with CTL activity (5–9). In this report we have shown that both i.n. and i.d. administration of protein with ISS-ODN leads to vigorous Th_1-biased systemic immune responses, whereas only i.n. delivery induces a mucosal immune response. Therefore, i.n. delivery of relevant antigens with ISS-ODN may well prove superior to i.d. delivery for the induction of protective

immunity to mucosal pathogens. Our personal experience has been that ISS-ODN is easy to manufacture, stable, and without i.d.entified toxicity at immunogenic doses in mice and primates (unpublished observations). Additionally, use of antisense phosphorothioate oligodeoxynucleotides in monkeys and human clinical trials has demonstrated no significant toxicity with daily doses of up to fivefold more per kilogram than those used in the present study (19). Moreover, we and others have shown that human and mouse immunocytes display similar immunologic responses to ISS-ODN, suggesting that our present findings might also be applicable to humans (8, 20). The data presented represent a proof of principle which shows that in addition to its systemic adjuvant activity, ISS-ODN is an excellent mucosal adjuvant, and suggests a novel approach for the development of vaccines against infectious agents.

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CpG Oligodeoxynucleotides Act as Adjuvants that Switch on T Helper 1 (Th1) Immunity

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Summary

Synthetic oligodeoxynucleotides (ODN) that contain unmethylated CpG motifs (CpG ODN) induce macrophages to secrete IL-12, which induces interferon (IFN)-γ secretion by natural killer (NK) cells. Since these cytokines can induce T helper 1 (Th1) differentiation, we examined the effects of coadministered CpG ODN on the differentiation of Th responses to hen egg lysozyme (HEL). In both BALB/c (Th2-biased) and B10.D2 (Th1-biased) mice, immunization with HEL in incomplete Freund's adjuvant (IFA) resulted in Th2-dominated immune responses characterized by HEL-specific secretion of IL-5 but not IFN-γ. In contrast, immunization with IFA-HEL plus CpG ODN switched the immune response to a Th1-dominated cytokine pattern, with high levels of HEL-specific IFN-γ secretion and decreased HEL-specific IL-5 production. IFA-HEL plus CpG ODN also induced anti-HEL IgG2a (a Th1-associated isotype), which was not induced by IFA-HEL alone. Control non-CpG ODN did not induce IFN-γ or IgG2a, excepting lesser increases in B10.D2 (Th1-biased) mice. Thus, CpG ODN provide a signal to switch on Th1-dominated responses to coadministered antigen and are potential adjuvants for human vaccines to elicit protective Th1 immunity.

Antigen-specific CD4+ Th cell responses can be divided into two types, type 1 and type 2, based upon cytokine secretion and effector function (1–3). Type 1 responses involve Th1 cells, whose differentiation is driven by IL-12 (from macrophages) and IFN-γ (from NK cells or T cells). Th1 cells secrete cytokines such as IFN-γ, IL-2, and lymphotoxin. In turn, IFN-γ activates macrophages and enhances immunoglobulin isotype switching to IgG2a, a hallmark of Th1 immunity (4). In contrast, type 2 responses involve IL-4–dependent differentiation of Th2 cells, which produce IL-4, IL-5, IL-10, and IL-13. Type 2 responses are associated with decreased macrophage activation, since some Th2-associated cytokines depress certain macrophage functions. The Th1/Th2 model provides a useful conceptual framework for Th differentiation, and the existence of distinct type 1 and type 2 responses is clearly established, although certain aspects of the model require further investigation (5). Moreover, differential induction of type 1 or type 2 responses is required for protective immunity to certain infectious diseases, and induction of the wrong response type can increase susceptibility to infection (see Discussion). Thus, the type of response induced by a vaccine may be crucial to its efficacy.

The type of Th response generated to an administered antigen can be directed by the type of adjuvant used. Injection of antigen in CFA induces a Th1-dominated response to the antigen, while injection of antigen in IFA induces a Th2-dominated response (6). However, because of its undesirable inflammatory side effects, CFA is not suited for use in human vaccines. Since type 1 immunity plays an important role in the protective response to infection with certain microbes, it is now important to characterize other novel adjuvants that safely induce type 1 immunity and that may potentially be incorporated in future human vaccines. The recent discovery that certain DNA preparations affect cytokine expression by cells of the innate immune system suggests the possibility that DNA preparations could be used as adjuvants to influence the differentiation of Th responses.

The ability of DNA to induce expression of cytokines depends on its source and characteristics (7). In vitro, bacterial DNA induces macrophage expression of IL-12 (8) and TNF-α (9), which are not induced by mammalian DNA. Bacterial DNA also indirectly activates NK cells and stimulates their production of IFN-γ (10–12), since NK cell production of IFN-γ is triggered by IL-12 that is generated by macrophages in response to bacterial DNA (8, 13).

To define components of bacterial DNA that have immunomodulatory effects, a panel of synthetic oligodeoxy-

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nucleotides (ODN)[1] was used to identify specific 6-base pair sequences that conferred activity (14). These sequences shared a CpG motif, containing a central unmethylated CpG dinucleotide preferentially flanked by two 5′ purines and two 3′ pyrimidines. CpG dinucleotides are present in bacterial DNA at the expected frequency of 1/16 bases, but they are three- to fourfold less frequent in mammalian DNA, a phenomenon known as CpG suppression (15). Also, the cytosines in CpG dinucleotides in mammalian DNA are highly methylated, whereas those in bacterial DNA are not (15). Elimination of the CpG sequence or methylation of the cytosine abrogates the stimulatory activity of ODN containing CpG motifs (CpG ODN) and bacterial DNA (9, 11, 14).

When added to splenocytes in culture, CpG ODN induce production of the Th1-associated cytokines IFN-γ and IL-12, as well as the Th2-associated cytokine, IL-6, within several hours (16). However, production of other Th2-associated cytokines, such as IL-4, IL-5, and IL-10, is not detected. The rapid production of IFN-γ is mediated by NK cells stimulated by IL-12 secretion from CpG-activated macrophages; this initial phase of IFN-γ production does not require T cells (8, 13). The induction of IFN-γ and IL-12 (which promote Th1 responses), but not IL-4 (which promotes Th2 responses), suggests that administration of CpG ODN in vivo might produce an environment favoring a Th1 immune response. Indeed, some bacterial plasmid DNA vaccines, which contain this CpG motif, cause development of antigen-specific CD4+ splenocytes that secrete IFN-γ, but not IL-4 or IL-5 (17, 18).

The effect of CpG ODN on antigen-specific T cell responses has not been previously tested. Our current experiments directly test the hypothesis that CpG ODN may serve as adjuvants to switch on Th1 responses. While immunization with hen egg lysozyme (HEL) in IFA induced a Th2-dominated response to HEL, immunization with IFA-HEL plus CpG ODN induced a strongly Th1-dominated response to HEL, as measured by production of specific IgG2a antibody and production of IFN-γ by antigen-stimulated T cells. We propose that CpG ODN function as adjuvants that switch on Th1 responses, making them important candidate adjuvants for potential use in future human vaccines.

Materials and Methods

Oligodeoxynucleotides. ODN were purchased from Operon Technologies (Alameda, CA) or Oligos Etc. (Wilsonville, OR). ODN were phosphorothioate-modified to increase their resistance to nuclease degradation. ODN used in these studies are listed in Table 1 and their sequences are given here (CpG motifs or reversed non-CpG motifs are underlined). Sequences of ODN that were phosphorothioate-modified throughout (S ODN) are: CpG ODN 1826, TCCATGACGTTCCTGACGTT; non-CpG ODN 1745, TCCAATGAGCTTCCTGAGTCT; CpG ODN 1760, ATAATCGACGTTCAAGCAAG; non-CpG ODN 1908, ATAATAGAGCTTCAAGCAAG. Sequences of ODN phosphorothioate-modified on the ends only (S-O ODN) are: CpG ODN 1585, GGGGTCAACGTTGAGGGGGG; and non-CpG ODN 1972, GGGGTCTGTGCTTTTGGGGGG. The first two 5′ end bonds and last five 3′ end bonds of the S-O ODN are phosphorothioate-modified. Synthetic ODN were dissolved in TE (10 mM Tris, 1 mM EDTA). LPS content of ODN was <1 ng LPS/mg DNA, as measured by Limulus amebocyte assay (QCL-1000; BioWhittaker, Walkersville, MD).

Immunizations. BALB/c and B10.D2 mice (Jackson Laboratory, Bar Harbor, ME and Harlan Sprague Dawley, Indianapolis, IN) were housed in microisolators under specific pathogen-free conditions and injected at 7–12 wk of age. HEL (Sigma Chem. Co., St. Louis, MO) was dissolved in PBS, ODN were dissolved in TE, and LPS (*E. coli* 0127:B8; Difco, Detroit, MI) was dissolved in PBS. ODN were added to HEL in a volume less than 10% of the final volume. HEL solutions with or without ODN or LPS were combined with IFA (GIBCO BRL, Gaithersburg, MD) at a 1:1 (vol/vol) ratio and emulsified to achieve a final HEL concentration of 1 mg/ml. CFA was prepared by suspending *Mycobacterium tuberculosis* H37 RA (Difco) at 4 mg/ml in IFA, and CFA was emulsified with the HEL solutions as above. Groups of three mice were injected i.p. with 200 μl of an emulsion and killed 3 wk after injection.

ELISA Assay for Antigen-specific Antibody Production. Sera were collected from mice by tail bleed 3–4 d before sacrifice (15–18 d after immunization with HEL), then diluted 1:10 in PBS/0.2% sodium azide and stored at −20°C. For ELISA, Nunc brand 96-well immunoplates (Fisher, Pittsburgh, PA) were coated by overnight incubation at 4°C with HEL at 10 μg/ml in 0.1 M sodium bicarbonate buffer. Plates were washed and blocked with PBS with 0.05% Tween (PBST) containing 0.1% gelatin for 1–2 h at room temperature. Sera were added to the top row of each plate and serial 1:3 dilutions in PBS were then made into subsequent rows. The plates were incubated overnight at 4°C and washed. Alkaline phosphatase-conjugated detecting antibody was added in PBST/0.1% gelatin and incubated for 2 h at room temperature. For IgG1 and IgG2a detection, goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was used at 1:4,000. For total Ig detection, goat anti-mouse Ig(H+L) (Southern Biotechnology Associates), specific for IgM + IgG + IgA, was used at 1:2,000. The colorimetric assay was developed with para-nitrophenyl phosphate (50 mg/ml in 2.5 M sodium bicarbonate/2.5 M magnesium chloride buffer) for 1–3 h. Absorbance at 405 nm was determined using a Beckman Bio Tek Microplate Autoreader (EL309; Beckman Instruments, Palo Alto, CA). The serum from each mouse was assayed in duplicate and the mean value was used to represent each animal. These values were used to calculate the mean and standard deviation for each group of three mice.

ELISA Spot Assay for Cytokine Production. Splenocytes were isolated from mice 3 wk after immunization. A modified ELISA spot assay for detection of cytokine production by splenocytes has been developed in prior work (6). ELISA spot plates (Polyfiltronics, Rockland, MA) were coated with capture antibody for IFN-γ (R46A2; 4 μg/ml in PBS) or IL-5 (TRFK5; 5 μg/ml in PBS) overnight at 4°C. Plates were then washed and blocked with PBS/1% BSA for 1–2 h at room temperature. After washing, freshly isolated splenocytes were plated at 10^6 cells/well in serum-free medium, HL-1 (BioWhittaker), supplemented with

[1] *Abbreviations used in this paper:* HEL, hen egg lysozyme; ODN, oligodeoxynucleotides; PBST, PBS with Tween; S ODN, phosphorothioate-modified ODN; S-O ODN, ODN with partial phosphorothioate modification.

Table 1. *Sequences of Synthetic ODN*

ODN	Sequence*	Motif	Backbone
	↓↓ ↓↓		
1826	TCCATGACGTTCCTGACGTT	CpG	S ODN
1745	TCCAATGAGCTTCCTGAGTCT	non-CpG	S ODN
	↓↓ ↓↓		
1760	ATAATCGACGTTCAAGCAAG	CpG	S ODN
1908	ATAATAGAGCTTCAAGCAAG	non-CpG	S ODN
	↓↓		
1585	GGGGTCAACGTTGAGGGGGG	CpG	S-O ODN
1972	GGGGTCTGTGCTTTTGGGGGG	non-CpG	S-O ODN

Nucleotides that have their 3′ linkage phosphorothioate modified to increase resistance to nuclease degradation are in bold print.
*The CpG motifs or corresponding non-CpG motifs are underlined.

L-glutamine and penicillin/streptomycin, in the presence or absence of HEL (100 μg/ml). In some experiments purified GK1.5 anti-CD4 antibody (American Type Culture Collection, Rockville, MD) was added at 10–30 μg/ml to block CD4 T cell function. After culture for 24 h (for IFN-γ detection) or 48 h (for IL-5 detection), cells were removed by washing with PBS and then PBST. Detecting antibody (XMG1.2-HRP, 1:400 for IFN-γ; TRFK4, 4 μg/ml for IL-5) was added in PBST/1% BSA and incubated overnight. For the IL-5 assay only, anti-IgG2a-HRP (Zymed, South San Francisco, CA) was added after washing with PBST, and the plates were incubated for 2 h at room temperature. All plates were washed with PBS before developing the colorimetric assay by the addition of 1% 3-amino-9-ethylcarbazole/*N,N*-dimethylformamide in 0.1 M sodium acetate buffer (1:30 vol/vol) for 45–60 min. The plates were then washed with distilled water and air dried. Spots were quantitated by an image analysis program (Optimas, Bothell, WA).

Results

Coadminstered CpG ODN Induce Production of HEL-specific IgG2a (a Th1-associated Isotype). BALB/c mice were injected i.p. with 200 μg of HEL in the following adjuvants: CFA, IFA, IFA with CpG ODN 1826, or IFA with a similar ODN lacking the CpG motif, ODN 1745. ODN 1826 and ODN 1745 are phosphorothioate-modified for the entire length of the backbone (S ODN; see Table 1), which greatly increases resistance to nuclease degradation (19). Based on preliminary dose titration studies, ODN were initially used at 100 μg/mouse. Sera were collected 15–18 d after immunization and assayed for anti-HEL Ig (total or specific isotype) by ELISA. Consistent with previous results demonstrating that IFA induces a Th2 response while CFA induces a Th1 response to antigen (6), mice injected with IFA-HEL did not produce detectable IgG2a responses (Fig. 1 *A*). In contrast, mice injected with CFA-HEL produced high levels of IgG2a. The addition of non-CpG ODN 1745 to the IFA-HEL protocol did not induce IgG2a production. However, immunization with IFA-HEL-CpG ODN 1826 altered the isotype profile of the antibody response, causing a marked increase in anti-HEL IgG2a. Furthermore, in three independent experiments, the production of HEL-specific IgG2a was consistently higher in mice treated with IFA-HEL-CpG ODN 1826 than in mice treated with CFA-HEL.

Despite the changes in IgG2a responses, similar levels of anti-HEL IgG1 or total anti-HEL Ig were produced by all immunizations (Fig. 1, *B* and *C*). Thus, immunization with IFA-HEL or IFA-HEL-non-CpG ODN 1745 was successful and sufficient to generate an antibody response to HEL, with both anti-HEL IgG1 and total anti-HEL Ig levels comparable to those seen with CFA-HEL or IFA-HEL-CpG ODN 1826. Although the IgG1 isotype has been linked to Th2 responses, our data demonstrate that IgG1 can also be observed in Th1-dominated responses and, at least in this system, cannot be used to accurately assess Th differentiation. We conclude that the increased IgG2a production associated with IFA-HEL-CpG ODN 1826, like that caused by CFA-HEL, represents a selective induction of this isotype, i.e., a qualitative switch in the relative levels of antibody isotypes produced rather than a simple enhancement of all anti-HEL isotypes.

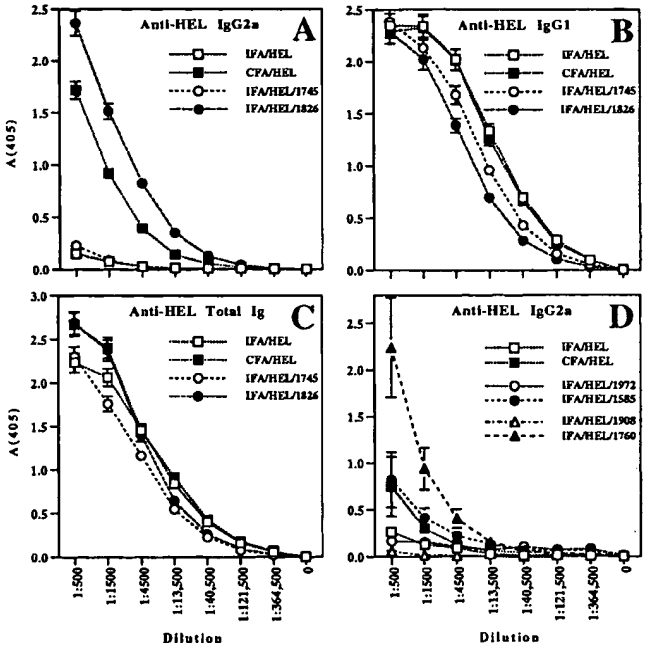

Figure 1. Th1-associated antigen-specific IgG2a responses are induced by immunization of BALB/c mice with IFA-HEL-CpG ODN but not IFA-HEL-non-CpG ODN. (*A–C*). Mice were injected i.p. with CFA-HEL (a control for a Th1-dominated response), IFA-HEL (a control for a Th2-dominated response), or IFA-HEL with 100 μg of CpG ODN 1826 or non-CpG ODN 1745. Sera were collected from mice 15–18 d after injection and assayed by ELISA for: (*A*) anti-HEL IgG2a, an isotype associated with Th1-dominated responses; (*B*) anti-HEL IgG1; and (*C*) anti-HEL total Ig response. *A–C* represent data from a single experiment representative of three similar experiments. (*D*) BALB/c mice were immunized as above, except that 30 μg of CpG ODN 1585, non-CpG ODN 1972, CpG ODN 1760, or non-CpG ODN 1908 was used for each mouse. Anti-HEL IgG2a antibodies were detected by serum ELISA. Data shown in *D* are representative of three similar experiments.

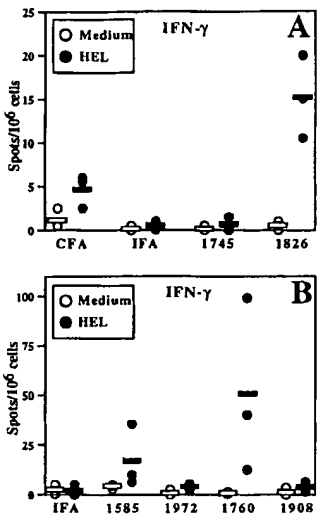

Figure 2. CpG ODN enhance HEL-specific IFN-γ production by BALB/c splenocytes. Mice were immunized as in Fig. 1 with 100 μg ODN/mouse in *A* and 30 μg ODN/mouse in panel B. After 3 wk, splenocytes were isolated and incubated with HEL (*closed circles*) or medium alone (*open circles*). ELISA spot assay was performed and spots were quantitated by a computerized image analysis program. Each point represents the mean number of spots per well for one mouse (assayed in duplicate); horizontal bars indicate the mean of points for each group of mice. Similar results were observed in five independent experiments with CpG- and non-CpG ODN in BALB/c mice.

To confirm the role of the CpG motif, we also examined the effects of two additional pairs of CpG and non-CpG ODN. CpG ODN 1760 and a related non-CpG control, ODN 1908, are S ODN (Table 1). CpG ODN 1585 and a related non-CpG control, ODN 1972, are phosphorothioate-modified on the ends only (S-O ODN; see Table 1). ODN 1760 and ODN 1826 share a common CpG motif, GACGTT. Immunization of BALB/c mice with HEL in IFA with or without 30 μg of each ODN showed that both CpG ODN (ODN 1760 and ODN 1585) induced anti-HEL IgG2a antibodies, which were not induced by the non-CpG ODN (ODN 1908 and ODN 1972) (Fig. 1 *D*). The S ODN 1760 induced significantly higher levels of anti-HEL IgG2a than CFA or the S-O ODN 1585 (which is more nuclease-sensitive than ODN 1760). Anti-HEL IgG1 and total anti-HEL Ig responses were similar in all groups (data not shown), again indicating that all of the immunizations generated anti-HEL antibody responses of similar overall magnitude. We conclude that antigen-specific IgG2a antibodies are induced by CpG ODN, suggesting that CpG ODN induce a Th1-dominated response to coadministered protein antigen.

Coadministration of CpG ODN Induces Th1-dominated Antigen-specific Cytokine Responses. We used a modified ELISA spot assay (see Materials and Methods) to assess recall antigen-specific IFN-γ secretion as a measure of Th1 memory cells induced after immunization with HEL. Three weeks after immunization of BALB/c mice with HEL in adjuvant, splenocytes were isolated, incubated in vitro with or without HEL and assayed for IFN-γ production. Upon restimulation with HEL, splenocytes from mice immunized with IFA-HEL showed little or no antigen-specific production of IFN-γ (Fig. 2 *A*), as expected (6). In contrast, splenocytes from mice immunized with CFA-HEL showed antigen-specific production of IFN-γ, demonstrating that CFA-HEL induced a Th1 response to HEL in these mice, as previously observed (6). Prior studies using isolated spleen-derived CD4+ T cells (with irradiated BALB/c-*scid* splenocytes as antigen-presenting cells) have shown that antigen-specific cytokine secretion measured by this assay is mediated by CD4+ T cells (Yip, H., A. Karulin, M. Tary-Lehmann, P. Heeger, R. Trezza, T. Forsthuber, and P.V. Lehmann, manuscript submitted for publication).

The results obtained with ODN established an important role for the CpG motif in determining Th differentiation. Immunization with IFA-HEL-non-CpG ODN 1745 did not enhance antigen-specific IFN-γ production over that observed with IFA-HEL. In contrast, immunization with IFA-HEL-CpG ODN 1826 strongly induced the production of antigen-specific cells secreting IFN-γ (Figs. 2 *A* and 3). Immunization with IFA-HEL-CpG ODN 1826 produced two- to fourfold more antigen-specific IFN-γ secretion than observed with CFA-HEL (Fig. 2 *A* and data not shown).

To determine the dose range for effective Th1 adjuvant activity of CpG ODN 1826, BALB/c mice were injected i.p. with 200 μg HEL in IFA, together with 0, 10, 30, or 100 μg ODN 1826. Antigen-specific serum Ig levels were assayed as above. Production of anti-HEL IgG2a was strongly enhanced in mice treated with as little as 10 μg ODN 1826, while specific production of total Ig and IgG1 was not affected by ODN 1826 at any dose (data not shown). ELISA spot analysis of splenocytes from these mice showed strong induction of IFN-γ by 30 or 100 μg ODN 1826 and lesser enhancement with only 10 μg (data not shown). Thus, Th1-directing adjuvant activity of CpG ODN is seen with doses as low as 10 μg in BALB/c mice.

To confirm that the Th1 adjuvant activity of CpG ODN 1826 was specific to the CpG motif, other CpG and non-CpG ODN were examined for effects on the differentiation of the Th response to HEL. As demonstrated in Fig. 2 *B*, immunization with both of the additional CpG ODN (ODN 1760 and ODN 1585) increased the number of cells secreting IFN-γ in response to secondary stimulation with HEL, while little or no increase was seen with the non-CpG ODN (ODN 1972 and ODN 1908). Consistent with the pattern of IgG2a induction, the S ODN 1760 appeared to have a greater effect than the S-O ODN 1585 on increasing numbers of IFN-γ–secreting cells. Thus, the number of cells secreting IFN-γ is enhanced by CpG ODN but not by non-CpG ODN, supporting the induction of Th1-dominated responses by CpG ODN. Furthermore, the addition of anti-CD4 antibody (GK1.5 at 10–30 μg/ml) during the in vitro antigen stimulation blocked CpG ODN-enhanced, HEL-specific IFN-γ secretion (data not shown), confirming that the CpG ODN-enhanced production of IFN-γ was T cell–dependent in this system.

To assess Th2 differentiation, ELISA spot analysis was similarly performed to detect splenocytes producing IL-5 (Fig. 4). Immunization with IFA-HEL induced cells that secreted IL-5 in response to restimulation with HEL, consistent with a Th2-dominated response. In contrast to the results with IFA-HEL, little or no HEL-specific IL-5 secretion was seen in mice immunized with CFA-HEL, consistent with a Th1-dominated anti-HEL response in these mice. HEL-specific IL-5 secretion was observed after im-

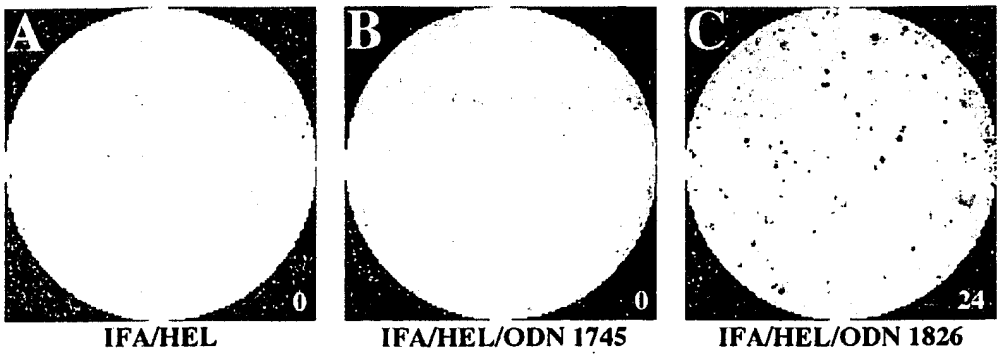

Figure 3. ELISA spot assessment of IFN-γ production by splenocytes from immunized BALB/c mice. Pictures show representative images of ELISA spot wells from the experiment shown in Fig. 2 *A*. The number of spots, as quantitated by an image analysis program, is indicated next to each well. Each well contained HEL (100 μg/ml) and 10^6 splenocytes isolated from mice immunized with IFA-HEL (*A*), IFA-HEL-non-CpG ODN 1745 (*B*) or IFA-HEL-CpG ODN 1826 (*C*).

munization with IFA-HEL-non-CpG ODN (e.g., ODN 1745 and ODN 1972), although immunization with IFA-HEL-non-CpG ODN 1745 produced somewhat lower levels of IL-5 than observed with IFA-HEL. In contrast, greater reduction in HEL-specific IL-5 secretion was observed after immunization with CpG ODN (ODN 1826, ODN 1760 and ODN 1585). Thus, the addition of CpG ODN induced a switch from a Th2-dominated response to a Th1-dominated response, as manifested by a decrease in Th2-associated cytokine secretion as well as the induction of Th1-associated cytokine secretion.

Together, these results indicate that CpG ODN directed Th1 differentiation in the T cell responses to coadminstered antigen. Relative to immunization with IFA-HEL, immunization with IFA-HEL-CpG ODN increased HEL-specific IFN-γ production by splenocytes, decreased HEL-specific IL-5 production by splenocytes and increased IgG2a anti-HEL titers. Furthermore, the Th1 adjuvant activity of CpG ODN for both antigen-specific antibody and cytokine production was significantly greater than that of an established Th1 adjuvant, CFA.

CpG ODN Direct Th1-dominated Responses in Th1-biased (B10.D2) Mice as well as Th2-biased (BALB/c) Mice. Strains of mice differ in genetic bias toward the development of Th1- or Th2-dominated Th responses. Earlier publications have demonstrated that BALB/c mice are Th2-biased, while B10.D2 mice are more Th1-biased (20). To explore the impact of varying Th1/Th2 bias on the effect of CpG ODN, B10.D2 mice were injected i.p. with IFA-HEL, with or without 30 μg CpG ODN 1826 or non-CpG ODN 1745, and splenocytes were subsequently isolated for ELISA spot analysis. Immunization with IFA-HEL-CpG ODN 1826 produced a very high level of HEL-specific IFN-γ production, while IFN-γ was not produced after immunization with IFA-HEL alone (Fig. 5). Again, CpG ODN 1826 induced levels of HEL-specific IFN-γ production that exceeded even those seen after immunization with CFA-HEL, and augmentation of IFN-γ production, albeit at lower levels, was seen in B10.D2 mice treated with as little as 3 μg of ODN 1826 in IFA-HEL (data not shown). Immunization with IFA-HEL plus either of the two other CpG ODN, ODN 1760, and ODN 1585, also induced HEL-specific secretion of IFN-γ (data not shown). Immunization with the non-CpG ODN 1745 and 1908 (30 μg dose) induced HEL-specific production of IFN-γ by splenocytes from B10.D2 mice, but at a minimal level (Fig. 5 and data not shown), while the other non-CpG ODN, ODN 1972, did not induce IFN-γ (data not shown). Thus, CpG ODN had strong Th1 adjuvant activity in Th1-biased as well as Th2-biased mice, while non-CpG ODN induced little or no Th1 differentiation, as assessed by antigen-specific secretion of IFN-γ.

The effects of CpG ODN on IFN-γ responses were paralleled by changes in IgG2a levels in B10.D2 mice. Again, immunization with IFA-HEL-CpG ODN 1826 (3, 10, or 30 μg ODN) induced high titers of anti-HEL IgG2a, and similar results were seen with the other CpG ODN, ODN

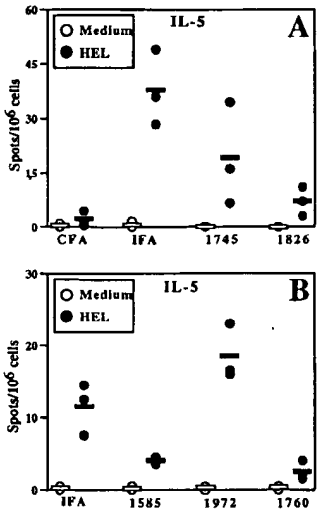

Figure 4. CpG ODN decrease HEL-specific IL-5 production by BALB/c splenocytes. Mice were immunized as in Fig. 2 (30 μg ODN/mouse), and splenocytes were harvested for in vitro restimulation with or without HEL. ELISA spot analysis was performed for IL-5. The data are representative of five similar experiments with CpG- and non-CpG ODN in BALB/c mice.

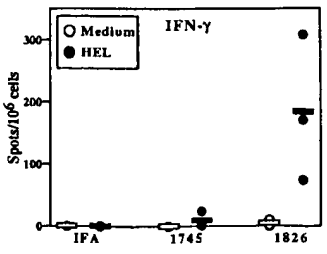

Figure 5. Induction of HEL-specific IFN-γ responses by CpG ODN in B10.D2 mice. B10.D2 mice were immunized as in Fig. 1, except that ODN were used at 30 μg per mouse. Three weeks after immunization, HEL-specific production of IFN-γ by splenocytes was measured by ELISA spot assay as in Fig. 2. The data shown are representative of three similar experiments.

1760, and ODN 1585 (only the 30 μg dose was assessed, data not shown). Mice treated with 30 μg non-CpG ODN 1745 also produced anti-HEL IgG2a, though the levels were not as high as in mice treated with ODN 1826, but the other non-CpG ODN (ODN 1908 and ODN 1972) did not induce IgG2a production. Anti-HEL total Ig and IgG1 levels were similar under all of these conditions (data not shown). These results confirm that CpG ODN enhance Th1-associated antigen-specific IgG2a responses in both Th1- and Th2-biased mouse strains.

Discussion

Because it is highly effective in inducing both cellular and humoral immunity, CFA has been an important model adjuvant (21). Furthermore, CFA has been shown to induce Th1-dominated immune responses (6). However, due to its inflammatory side effects, CFA cannot be used in humans. Thus, the discovery and characterization of adjuvants that promote Th1 cell-mediated immunity is currently an important area in vaccine development. Our results establish that CpG ODN are excellent candidate adjuvants for vaccines to induce Th1 immunity.

CFA is prepared by mixing two components, IFA (mineral oil) and nonviable *Mycobacterium tuberculosis*. As an adjuvant, CFA has been proposed to provide two crucial functions. First, it creates a local antigen depot (by entrapment of antigen in the mineral oil emulsion) which allows for prolonged regional antigenic stimulation. This function is also provided by IFA. Second, CFA contains immunomodulatory substances derived from *Mycobacterium tuberculosis* that stimulate immune responses and promote Th1 differentiation. It is possible that the adjuvant activity of CFA may be due in part to mycobacterial DNA, as *M. bovis* DNA sequences have been shown to be immunostimulatory (22, 23). Thus, a general strategy for the development of type 1 vaccine adjuvants may be to provide both an antigen depot and an immunomodulatory function that promotes Th1 differentiation.

Our studies address the ability of CpG ODN to modulate the differentiation of Th responses. In these experiments, ODN were mixed with IFA, which itself establishes an antigen depot but does not promote Th1 differentiation (future vaccines using CpG ODN may include alternative components such as biodegradable oils to optimize vaccine safety and efficacy). Mice that were immunized with IFA-HEL developed both humoral and Th2 cellular immune responses, consistent with prior observations (6). However, the addition of CpG ODN to this system induced strong Th1-dominated responses. Th1-dominated responses were induced with as little as 3 μg CpG ODN in B10.D2 mice and 10 μg CpG ODN in BALB/c mice.

As a direct measure of Th1 differentiation, we monitored the number of splenocytes secreting IFN-γ after immunization and in vitro stimulation with HEL by ELISA spot assay. This assay has been shown to detect cytokine secretion by individual CD4+ T cells (Yip, H., A. Karulin, M. Tary-Lehmann, P. Heeger, R. Trezza, T. Forsthuber, and P.V. Lehmann, manuscript submitted for publication). Also, addition of a blocking anti-CD4 mAb to the splenocytes during in vitro stimulation with HEL decreased HEL-specific production of IFN-γ by >80% (data not shown), confirming that the production of IFN-γ was dependent on antigen-specific CD4+ T cells. We also measured IFN-γ production by standard ELISA and found the same pattern of results as obtained by the ELISA spot assay (data not shown).

The addition of CpG ODN as adjuvants produced levels of IFN-γ that surpassed even the levels observed with CFA. Two of the non-CpG ODN (ODN 1745 and ODN 1908) induced IFN-γ secretion, but only at minimal levels and only in Th1-biased B10.D2 mice. ODN 1745 also induced production of antigen-specific IgG2a, although ODN 1908 did not. The slight CpG-like effects of ODN 1745 may be due in part to the presence of TG dimers in a context that provides a weak analogue of a CpG motif. Other studies have shown that weak induction in vitro of other CpG-like effects by ODN 1745 (B cell proliferation, secretion of IL-6, TNF-α, and IL-12) are eliminated in analogues of ODN 1745 that lack TG dimers (Krieg, A.M., unpublished observations). In addition, weak CpG-like effects could be triggered by the DNA backbone of non-CpG ODN (e.g. ODN 1745 and ODN 1908), since the phosphorothioate backbone of modified ODN has some intrinsic immunostimulatory properties (7, 24). Despite minor activities of some non-CpG ODN, the CpG ODN were vastly superior for inducing Th1-dominated immune responses in both Th1- and Th2-biased mouse strains. These observations indicate that the CpG motif provides a strong and reliable stimulus for Th1 differentiation in animals of differing genetic background.

We also assessed the differentiation of Th responses indirectly by the isotype profile of antigen-specific antibody responses. CpG ODN induced levels of antigen-specific IgG2a that surpassed even those achieved with CFA. Total anti-HEL Ig and anti-HEL IgG1 levels induced by CpG ODN did not differ appreciably from those induced by IFA or CFA, indicating that all of the immunization protocols used in this study were successful and effective for generation of anti-HEL Ig. Thus, the increased production of IgG2a induced by CpG ODN and CFA represents a qualitative switch in Ig isotype production from an IFA-induced Th2-influenced pattern to a Th1-influenced pattern.

We assessed the differentiation of Th2 responses by antigen-specific IL-5 production. Although IL-4 is also produced during Th2 responses, there can be differences in the production and source of these two cytokines under some circumstances. Even in the context of a Th1 response, antigen-stimulated T cells can induce IL-4 (but not IL-5) secretion by non-T cells (Karulin and Lehmann, unpublished observations). Other groups have reported that treatment with IL-12 at the time of immunization can induce antigen-specific IL-4 production by splenocytes (25), again demonstrating that IL-4 can be detected alongside markers of Th1

responses. Therefore we chose IL-5 secretion as a clear, specific marker for Th2 differentiation in our studies, i.e., a cytokine whose antigen-triggered secretion could be attributed to antigen-specific T cells. The differentiation of cells secreting IL-5 was monitored by the ELISA spot assay. We also tested the supernatants from similar incubations for IL-5 by standard ELISA, which revealed the same pattern of results (data not shown).

ELISA spot analysis demonstrated that HEL-specific IL-5 secretion was induced by immunization with IFA-HEL but was absent after immunization with CFA-HEL. Immunization with IFA-HEL-CpG ODN produced significantly lower levels of IL-5 secretion than observed with IFA-HEL. Thus, in addition to enhancing Th1 differentiation, CpG ODN appear to at least partially switch off Th2 differentiation to produce Th1-dominated immune responses. Moreover, these studies examined Th differentiation after a single immunization. It is possible that repeated administration of antigen with CpG ODN would produce an even more polarized Th1 response.

The experiments shown here monitored IgG2a levels at 15-18 d after immunization and T cell responses at three weeks after immunization. Additional experiments also examined cytokine responses upon antigenic restimulation of T cells at 4 and 6 wk after immunization, as well as IgG2a levels at 3–4 d before these times. At all of these time points the exact same pattern of results was consistently found. For example, CpG ODN produced enhanced IFN-γ and IgG2a responses in all experiments at these later time points (data not shown). These results indicate that the pattern of T cell differentiation induced by a single immunization with CpG ODN remains stable for at least 6 wk.

The possibility that the effects of CpG ODN were due to contaminating LPS was excluded by the following observations. First, LPS levels in the ODN preparations were very low (<<1 ng LPS/mg DNA). Second, we immunized with IFA-HEL plus LPS at 1 ng/mouse (an amount 10–100-fold higher than the maximum amounts contributed by the highest ODN doses) and failed to see any increase in Th1-associated results (IFN-γ secretion and induction of IgG2a) or decrease in Th2-associated IL-5 secretion (data not shown).

One concern for DNA adjuvants, as with all adjuvants, is the potential for toxicity. The administration of bacterial DNA or stimulatory ODN can induce TNF-α release and fatal shock in mice that have been previously sensitized with D-galactosamine (26), and CpG DNA can prime mice for the Shwartzmann reaction (12). With regard to ODN adjuvants, mice given repeated high doses of CpG ODN develop a dose-dependent splenomegaly and can develop other toxicity related to excessive immune stimulation, including death (27). However, significant toxicity has not been observed at the low doses of ODN used for adjuvant function in our current studies, where we observed no significant changes in mouse appearance, behavior, and body weight (measured at multiple points throughout the experiment) or spleen weight (measured at the time of sacrifice). We also noted that spleens from mice injected with CFA were more difficult to remove, presumably due to post-inflammatory peritoneal fibrosis, while spleens from mice injected with CpG ODN appeared normal. Additional studies of spleen and lymph node sizes at earlier time points after administration of CpG ODN alone, at the doses used here, revealed mild splenomegaly and hyperplasia of draining lymph nodes that was reversible within 10–14 d. Mice injected with a single dose of up to 1 mg of the S ODN used in these studies (100-fold higher than the effective adjuvant dose) showed no apparent systemic toxicity or change in feeding, grooming, physical activity or behavior. We conclude that CpG ODN provide potent adjuvant activity at doses that produce no dangerous toxicity.

In the context of vaccine development, the ability to direct Th1 or Th2 differentiation of antigen-specific immune responses has significant implications for therapy of various infectious and autoimmune diseases (28). In the case of certain infectious diseases, Th1-dominated immune responses are protective, while Th2-dominated responses are associated with disease susceptibility. For example, in murine leishmaniasis, Th2-biased mouse strains (e.g., BALB/c) make IL-4–dominated responses to parasite antigens and are susceptible, whereas mice that mount Th1-dominated responses involving IFN-γ secretion (e.g., C57BL/6) are resistant (29–31). Moreover, susceptible mice can be made resistant by administration of IL-12 to enhance Th1 immunity or antibody blockade of IL-4 (32-34), and resistant mice can be made sensitive by blockade of IL-12 (35). In similar circumstances in humans, the ability to direct vaccine-induced immunity towards Th1 responses will dictate the success of vaccination. Thus, CpG ODN may be useful as adjuvants to induce protective Th1 immunity.

In other circumstances the utility of CpG ODN may lie in their potential ability to redirect pathogenic Th2 responses to less harmful Th1 responses. For example, Th2-dominated responses appear to cause allergy, and recent data suggest that administration of CpG ODN may prevent or even reverse ongoing allergic reactions, presumably by redirecting a Th2-dominated response to allergen (which promotes IgE synthesis) to a Th1-dominated response (Kline, J., T. Businga, T. Waldschmidt, J. Weinstock, and A.M. Krieg, manuscript submitted for publication). Similarly, autoimmune diseases that are potentially Th2-associated, such as systemic lupus erythematosis, may be amenable to such Th1 therapy. Th1 therapy, however, is potentially associated with the danger of inducing Th1-mediated pathology, such as certain Th1-associated autoimmune diseases (e.g., experimental allergic encephalomyelitis and type I insulin dependent diabetes mellitus) (36).

The CpG motif has been proposed to act as a danger signal that warns of bacterial infection and activates immune defenses (37). Similarly, one function of therapeutic adjuvants may be to identify vaccine antigens as dangers to which the immune system should respond. Thus, a danger signal provided by CpG ODN may provide potent adjuvant function. Our studies demonstrate that CpG ODN are

extremely effective as adjuvants to induce Th1-dominated immune responses without significant toxicity. This property makes CpG ODN attractive as candidate adjuvants for potential use in human vaccines for the prevention or treatment of a wide range of infectious diseases and immune disorders.

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◆ TRENDS IN MOLECULAR MEDICINE ◆

Control of immune responses by gene immunization

Delphine J Lee, Maripat Corr and Dennis A Carson

The use of plasmid DNA to elicit immune responses has greatly increased our ability to skew the desired immune response to a particular antigen. DNA immunization elicits potent cell-mediated responses including humoral immunity as well as cytolytic T-lymphocyte immunity. This review will first discuss the overall immune response induced by naked DNA vaccination and will then summarize recent advances in basic research on DNA immunization, which have furthered our understanding of the role of DNA as an adjuvant as well as a carrier of genetic material. Subsequently, we will consider the possible mechanisms by which DNA immunization is able to induce such immune responses and how DNA immunization may be useful in both basic science research and also in future vaccine development in various disease processes. Finally, we will examine the advantages and disadvantages of DNA vaccines as well as safety issues. In conclusion, DNA vaccination shows promise in a number of areas including infectious diseases, allergy and cancer immunotherapies.

Key words: DNA immunization; immunostimulatory sequence; immunotherapy; T-helper cell 1 (Th1).

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Introduction

The finding that mouse muscle cells could express genes encoded by plasmid DNA (pDNA) injected intramuscularly (im) has led to an explosion of experiments using gene immunization (1). Gene immunization refers to the induction of an immune response to an antigenic protein encoded by the injected DNA. The pDNA is circular with an origin of replication and an antibiotic resistance gene, allowing it to be produced at high levels in *Escherichia coli* under selective pressure. The DNA is purified from *E. coli* and injected intradermally (id) or im without any associated proteins or lipids, thus it is termed 'naked' DNA immunization. The gene encoding the antigen is expressed under the control of a strong eukaryotic promoter, along with the appropriate termination and polyadenylation sequences for the mRNA transcript to be properly translated in the mammalian cells. DNA immunization elicits potent antibody, T-helper (Th) and cytolytic T-lymphocyte (CTL) responses. Another method not discussed here is biolistic DNA injection, which involves the complexing of pDNA to gold particles and injecting with a 'gene gun' into the epidermal and dermal layers (2–4).

Portrait of an immune response

While protein antigens may be degraded or cleared by antibodies, DNA vaccines allow host cells to take up the DNA and synthesize antigenic protein. Antigens synthesized by host cells via DNA immunization can also be released from cells to be endocytozed by professional antigen-presenting cells (APCs) and then enter the major histocompatibility complex (MHC) class II presentation pathway to stimulate $CD4^+$ Th lymphocytes. $CD4^+$ Th lymphocytes, which recognize

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Abbreviations and acronyms

APC	antigen-presenting cell
CEA	carcinoembryonic antigen
CTL	cytolytic T lymphocyte
DTH	delayed-type hypersensitivity
GM-CSF	granulocyte–macrophage colony-stimulating factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISS	immunostimulatory sequences
MHC	major histocompatibility complex
NK	natural killer (cell)
PCR	polymerase chain reaction
pDNA	plasmid DNA
PEM	polymorphic epithelial mucin
Th	T-helper (cell)

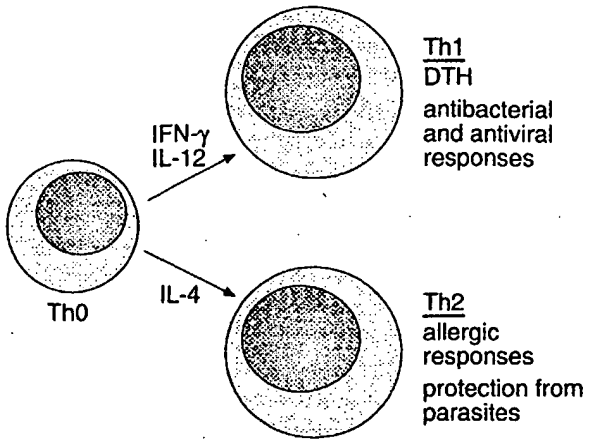

Figure 1. Cytokines present at the time of priming influence the differentiation of T-helper (Th) cells. The presence of interferon (IFN)-γ and interleukin (IL)-12 cause Th1 differentiation which is useful in delayed-type hypersensitivity (DTH), antibacterial and antiviral responses, while IL-4 induces Th2 differentiation which is responsible for allergic responses as well as for protection from parasites such as helminthic infections.

the antigenic peptide bound by MHC class II, become activated and then secrete cytokines to aid in the activation of other immune cells, such as B cells and cytotoxic T cells. Unlike standard protein-based vaccines, in addition to being presented on MHC class II, the intracellular source of antigen then has access to the MHC class I antigen presentation pathway. Antigens encoded by pDNA vaccines may utilize this intracellular pathway to elicit $CD8^+$ CTL responses subsequently. However, the mechanisms by which plasmid encoded antigens are presented via MHC class I molecules to naive $CD8^+$ T lymphocytes may be more complex. These will be considered in more detail when we discuss the mechanism by which gene immunization induces an immune response.

An important advantage of DNA vaccination is the type of immune response induced in $CD4^+$ Th cells. Th cells differentiate from Th0 precursors into two readily discernible populations, Th1 and Th2, on the basis of the types of cytokines they produce (5). Th1 cells produce interleukin (IL)-2, interferon (IFN)-γ and tumour necrosis factor (TNF)-β, while Th2 cells produce IL-4, IL-5, IL-6 and IL-13 (6). The dominant factors that determine such differentiation are the cytokines present during the priming period (7) (Fig 1). For example, if IFN-γ and IL-12 are present the resulting $CD4^+$ T cells will make IFN-γ, but if IL-4 is present the cells will make IL-4. Furthermore, Th1 and Th2 clones differ in the type of immune response they stimulate (Fig 1). Th1 cells mediate delayed-type hypersensitivity (DTH) reactions, increase immunoglobulin (Ig)G2a and IgG3 isotype synthesis (in the mouse) via IFN-γ secretion (8), and are associated with a strong CTL response. Th2 cells activate B cells to produce IgG1 and IgE subclasses via IL-4 secretion and activate eosinophils via IL-5 (9). The two polarized responses are cross regulating by the cytokines they secrete *in vivo*.

Most importantly, the type of Th immune response elicited in the setting of infection can greatly influence the outcome. For example, susceptible strains of mice infected with *Leshmania major* make a Th2 response, while surviving strains of mice make a Th1 response to infection and become immune (10, 11). Furthermore, in lesions of the resistant tuberculoid form of leprosy, mRNAs encoding for IL-2 and IFN-γ were most evident, while in the susceptible lepromatous form IL-4, IL-5 and IL-10 predominated (12). Also, it appears that highly resistant tuberculoid patients and healthy individuals exclusively develop Th1-like responses against mycobacterial antigen (13).

Naked pDNA immunization by id or im routes induces Th-cell differentiation to the Th1 type, which secrete high levels of IFN-γ and lead to the production of IgG2a antibodies as well as to the activation of strong MHC class I-restricted CTL responses (14–16). Not only can an ongoing antigen-specific Th2 response induced by protein in aluminum hydroxide (in alum) be subsequently 'switched' to an antigen-specific Th1 response by naked pDNA immunization, but the Th1 response induced by pDNA immunization also prevails over a later attempt to induce a Th2 response by protein in alum immunization (15).

Mechanism of action: antigen presentation

Immunization by either id or im routes results in antigen expression at the site of injection (1, 17). Cells transfected with the plasmid could theoretically present the antigen to activate a naive T cell, or the

antigen could be taken up and be presented by another cell. As MHC class II expression is mainly limited to cells of bone marrow origin, bone marrow-derived cells are likely candidates for presenting antigen to stimulate $CD4^+$ Th cells. On the other hand, MHC class I is present on all nucleated cells, so there is the possibility that cells other than conventional APCs may present antigen to activate $CD8^+$ T cells. Antigens presented on MHC class I are classically thought to be intracellular in origin; however, several studies have demonstrated exogenous antigens presented on MHC class I (18–23). Furthermore, the stimulation of a T cell via its antigen receptor in the absence of a second nonspecific costimulatory signal leads to tolerance rather than activation (24), although the nonspecific signal can be provided by another cell (25).

Therefore, in order for a MHC class I-restricted $CD8^+$ T cell to be primed by DNA immunization, either 1) a professional APC which expresses costimulatory molecules is directly transfected by the plasmid and presents antigen by the intracellular pathway; 2) a professional APC which expresses costimulatory molecules ingests protein produced by a somatic cell, processes and presents antigen by the exogenous pathway; 3) a directly transfected somatic cell processes and presents antigen by the intracellular pathway in an environment where an adjacent cell expresses the appropriate costimulatory molecules; or 4) a nonprofessional APC processes and presents antigen by the exogenous pathway in an environment where an adjacent cell expresses the appropriate costimulatory molecules. Studies using chimeric mice demonstrate that cells of haematopoietic origin are responsible for priming a CTL response following gene immunization (26). Furthermore, intraperitoneal injection of allogeneic myoblasts transfected *in vitro* can elicit a CTL response to the antigen restricted to the host's MHC haplotype (27). Because the myoblasts do not express the same MHC as the host, this demonstrates the capacity for APC presentation by protein transfer from somatic cells to APC. However, it is still unclear whether the bone marrow-derived APCs are directly transfected at the site of injection (Fig 2a) or ingest antigen produced by somatic cells transfected with the plasmid upon injection of naked pDNA (Fig 2b) (for review, see (28)).

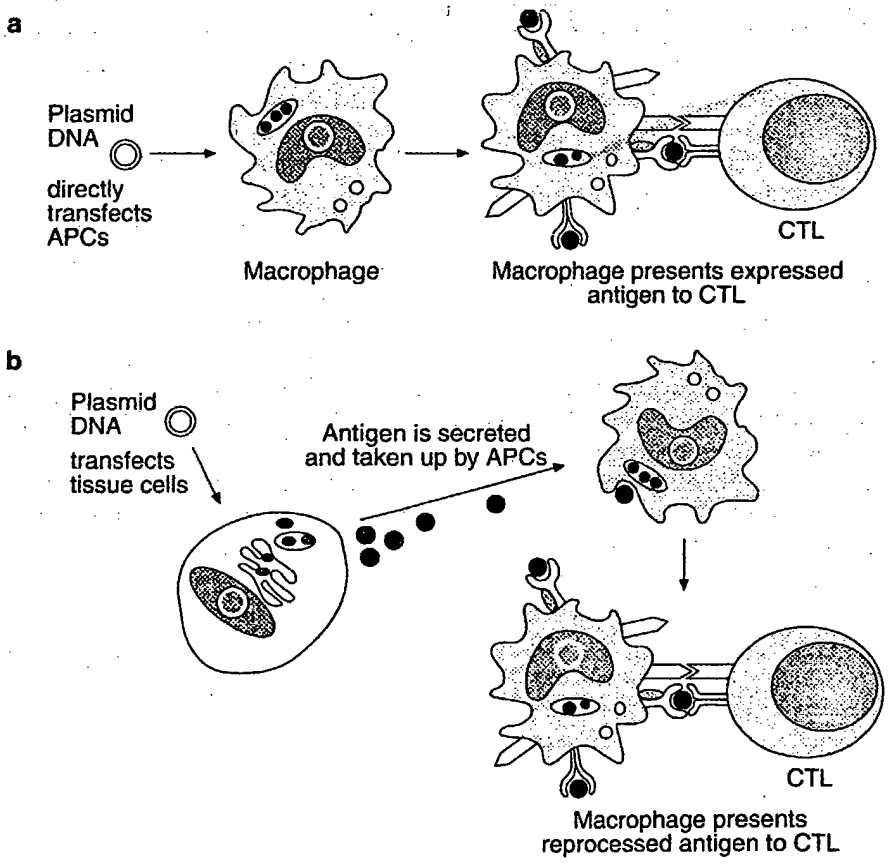

Figure 2. Two possibilities in the mechanism of cytolytic T lymphocyte (CTL) priming by DNA vaccination. (a) The bone marrow-derived antigen-presenting cells (APCs) are directly transfected by the injected plasmid DNA (pDNA) or **(b)** ingest antigen produced by somatic cells which were transfected with the pDNA upon injection.

Mechanism of action: induction of the Th1 response

The Th1 response to gene vaccination, characterized by increased IgG2a and IFN-γ with decreased IgE and IL-4, is largely due to the immunostimulatory effects of noncoding sequences present within the pDNA itself. Altering the number of immunostimulatory sequences (ISS) within the pDNA can change the magnitude of the Th1 response (29). In addition, coinjection of protein and oligonucleotides containing the ISS (30), or antigen combined with incomplete Freund's adjuvant and ISS-containing oligonucleotides (31), can also elicit an antigen-specific Th1 response.

The mechanism by which the ISS within the pDNA induce a Th1 response is unknown. The fact that fresh human monocytes/macrophages up-regulate expression of IFN-α, IFN-β, IL-12 and IL-18 mRNA *in vitro* upon transfection with ISS (30) suggests a role for soluble factors. All of these cytokines have been established as inducers of IFN-γ and promote the differentiation of naive Th0 cells to Th1 (32–35). It has been suggested that the Th1-inducing cytokines produced upon ISS stimulation may not only directly affect nearby Th0 cells but may also influence the cells of the innate arm of the immune system to produce IFN-γ to create an overall Th1-inducing environment.

However, despite the suggestive *in vitro* evidence of a role for cytokines from APCs such as monocytes/macrophages in the promotion of Th1 differentiation in DNA vaccination, the mechanism by which DNA vaccines induce an antigen-specific Th1 response *in vivo* is still not precisely defined. First of all, it is not clear whether Th cell priming occurs at the site of injection or at a nearby lymphoid organ. Antigen is expressed at the site of injection in myocytes by im injection (1) and in keratinocytes, fibroblasts and cells with the morphologic appearance of dendritic cells by id injection (17). pDNA was detected by polymerase chain reaction (PCR) in the tail but not in draining lymph nodes, spleen or liver 3 months after a single injection of 100 μg of pDNA (17). However, dendritic cells from the draining lymph nodes of mice immunized im with pDNA can elicit a cytokine response from an antigen-specific T-cell hybridoma. DNA extracted from these lymph node dendritic cells contained pDNA sequences detected by PCR (36). Similar results were obtained from Langerhan's cells (epidermal dendritic cells), which were allowed to emigrate *in vitro* from the skin of intracutaneously injected mice. However, direct detection of protein product in dendritic cells from the skin 0–5 h after id injection was unsuccessful. These studies suggest direct transfection of dendritic cells in both im and id injection.

One can envision several possibilities to explain these conflicting results. Data from Casares et al suggest that the APCs are directly transfected (36). If so, the APCs may be stimulated by ISS, make Th1-inducing cytokines and prime naive antigen-specific Th precursor cells to become Th1 cells (Fig 3). However, if Th priming occurs by a professional APC which takes up protein synthesized by transfected somatic cells, the mechanism for ISS induction of Th1 differentiation is less clear. CTL priming has been shown to require the site of injection to be intact in id immunization (M Corr, unpublished observations, 1995). This implies that the plasmid does not migrate out of the site of injection, and that either the CTL is primed directly at the site of injection or it is primed by protein processed by an APC in a lymphoid organ. If Th cells are primed peripherally at the site of injection, the role of soluble factors from ISS stimulation is clear. However, if the CTL priming occurs at a distant site from the ISS, it is difficult to envision the role for ISS-induced cytokines in the differentiation of Th1 cells after pDNA vaccination.

Another less likely mechanism is priming the Th cell by a somatic cell. ISS may induce expression of MHC class II and costimulatory molecules on a somatic cell either directly or indirectly by the inflammatory response induced by ISS. This change

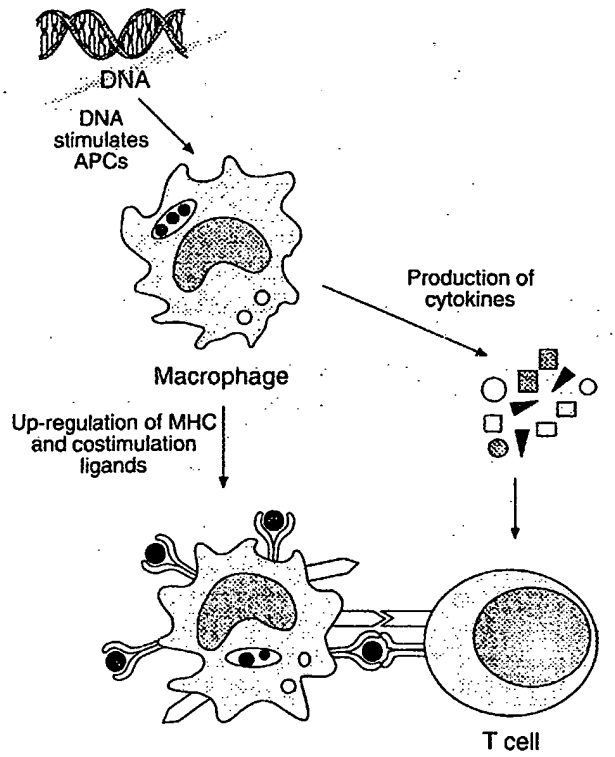

Figure 3. The two effects of plasmid DNA stimulation of antigen-presenting cells (APCs). First, transfection of incubation of APCs with DNA increases the secretion of proinflammatory cytokines which are T-helper cell 1-inducing, and secondly DNA activates APCs to up-regulate the expression of major histocompatibility complex (MHC) as well as costimulatory ligands to enhance T-cell activation.

may allow the somatic cell to become an APC to prime Th cells, at least during secondary immune responses. In addition, ISS-induced cytokine secretion by nearby APCs (or perhaps by other cells) could affect the Th-cell differentiation (Fig 3). In this regard, incubation of fresh mouse B cells, peritoneal macrophages or bone marrow-derived macrophages with ISS oligonucleotides, but not control oligonucleotides, has been shown to cause an up-regulation of a number of cell surface markers (E Martin-Orozco, unpublished observations, 1998) including costimulatory molecules which might influence the subsequent Th differentiation.

Evidence against direct transfection of monocyte/macrophages in naked DNA immunization either intramuscularly or intradermally comes from experiments utilizing a plasmid containing ISS which has the expression of the β-galactosidase gene under the macrophage scavenger receptor promoter (DJ Lee and M Corr, unpublished observations, 1996). This promoter is active in macrophages of various organs and tissues, particularly Kupffer cells, alveolar macrophages and macrophages in the spleen and lymph nodes (37). While the β galactosidase is expressed in macrophage cell lines *in vitro*, immunization *in vivo* gives neither antigen-specific antibody nor CTL responses. Coimmunization of this plasmid with another plasmid encoding granulocyte–macrophage colony-stimulating factor (GM-CSF) to induce differentiation of nearby monocytes does not significantly enhance the antibody nor the CTL response. This suggests that protein expressed by any directly transfected monocyte/macrophage is insufficient to generate an immune response.

A critical aspect of DNA immunization is the synthesis of antigen intracellularly, which potentially affords direct access to the MHC class I presentation pathway. An attractive hypothesis to explain the Th1-biased immune response in DNA immunization is the readily available presentation of antigenic epitopes on MHC class I and the subsequent stimulation of $CD8^+$ T cells. As $CD8^+$ T cells make IFN-γ, which can influence a naive $CD4^+$ T cell to differentiate to the Th1 phenotype, the $CD8^+$ T cells may be important. However, preliminary results suggest that $CD8^-$-deficient mice still make antigen-specific IgG2a comparable to wild-type mice in response to DNA immunization (DJ Lee, unpublished observations, 1997). Furthermore, neither β2m-dependent natural killer (NK) T cells which are absent in β2m –/– mice nor typical NK cells appear to affect the response to DNA immunization, as *in vivo* depletion of $NK1.1^+$ cells by antibody injection actually enhances the antigen-specific IgG2a (DJ Lee, unpublished observations, 1995). Gamma delta T cells have also been implicated in the regulation of Th1/Th2 development (38, 39). However, both wild-type mice and mice with a disrupted delta chain gene made comparable levels of antigen-specific IgG2a and IFN-γ to DNA immunization (DJ Lee and M Corr, unpublished observations, 1996).

In summary, the mechanisms of Th1 induction by DNA immunization are apparently complex. APCs such as monocyte/macrophages do make several Th1-inducing cytokines in response to the ISS present on the pDNA. However, direct evidence for the exact role of ISS-induced cytokines released by the APCs is lacking. Perhaps ISS affect the cell surface phenotype of APCs or the overall environment of the somatic tissue at the site of injection to promote Th1 differentiation.

Mechanism of action: persistence of the Th1 response

The Th1 response induced by pDNA immunization is regulated by CD4, CD8 or double negative ($CD4^-$/$CD8^-$) T cells, as adoptive transfer experiments demonstrate that the IgE-suppressive effect by DNA immunization can be transferred by either $CD4^+$ or $CD8^+$ T cells (40). However, studies by Hsu et al using DNA immunization to prevent the induction of IgE synthesis showed that passive transfer of $CD4^+$-depleted splenocytes but not $CD8^+$-depleted splenocytes were able to suppress the IgE response in rats (41). Interestingly, the IgG2a response was unchanged without regard to whether $CD4^+$ or $CD8^+$ cells were depleted. However, adoptive transfer of $CD4^+$ but not $CD8^+$ cells augments the IgG2a response to a subsequent β galactosidase in alum immunization (DJ Lee, unpublished observations, 1995). Furthermore, Manickan et al have demonstrated that the Th1-protective immune response to herpes simplex virus induced by DNA immunization is mediated by $CD4^+$ cells (14). These differences may be due to the experimental model, design and/or the time points at which the animals were tested. Nevertheless, all the data show that the dominance of a Th1 response induced by DNA immunization prevails in subsequent protein immunizations and can be transferred by T cells.

Applications

The ease with which antigens can be manipulated by altering constructs allows the use of different forms of an antigen, such as secreted or membrane bound or even one with deleted sequences, to avoid the expression of unwanted antigenic epitopes. Furthermore, while immunization by standard protein injections allows the cells of the immune system to take up and present the foreign protein, gene immunization gives the added advantage of ectopically expressing membrane-bound molecules, such as potential antigen-

presenting molecules or costimulatory ligands, which may alter the overall immune response. In studies with two separate antigen systems (ovalbumin and β galactosidase), gene immunization with combinations of plasmids expressing antigen and costimulators has proved to be effective in enhancing different arms of the immune system, with B7.1 being useful for cytotoxic T-cell priming and B7.2 enhancing antibody responses (42, 43). The expression of the costimulator ligand appears to act locally and is dependent on the presence of the plasmid expressing the costimulator molecule at the same site as the antigen-encoding plasmid.

By using DNA immunization, immune responses cannot only be augmented but also skewed. A co-injection strategy can be used to express ectopically the antigen-presenting molecule of interest (CD1d1) as well as the antigen (44). By using a combination of plasmids, DNA vaccination can therefore be used as a tool to prime a specific response not only to the antigen of interest, but it also allows us to restrict the immune response to the desired antigen-presenting molecule. For example, antigen-specific murine CD1-restricted lymphocytes can be generated *in vivo* by DNA immunization of normal mice with a combination of plasmids encoding chicken ovalbumin, murine CD1d and costimulatory molecules.

The simplicity of mixing different plasmids may also allow us to modulate an immune response by coexpressing soluble cytokines. GM-CSF, which may affect antigen presentation, stimulates both Th and B-cell responses in a number of systems (45–47).

Infectious diseases

Although antibodies may be helpful in neutralization of extracellular pathogens, cell-mediated immunity is needed to detect and destroy cells infected with viruses or other intracellular bacteria. For example, influenza viruses mutate in their envelope genes and as a result are able to evade the previous year's vaccine containing protein subunits that are directed at the envelope glycoprotein. The nucleoprotein of influenza is an internal viral protein and is less subject to the antibody-induced antigenic drift than the surface glycoproteins. Early studies of naked DNA vaccine applications utilized plasmids encoding the influenza internal core proteins and/or the surface glycoproteins in several animal models including mice (48), chickens (49) and ferrets (50) and have demonstrated protection among different viral strains with varying degrees of efficacy.

DNA vaccines have also been developed for the production of *in vivo* immunity against HIV-1. Chimpanzees can maintain protective immune responses to HIV-1 up to 48 weeks after challenge (51); however, other challenge models in macaques have produced partial protection at best (52). Preliminary studies from the first human clinical studies with DNA vaccine reveal little clinical or laboratory adverse events, and antibody responses as well as some increased cellular responses were also observed (52). Other examples of infectious diseases in which DNA vaccines are being developed include malaria (53), tuberculosis (54), hepatitis B virus (55, 56) and hepatitis C virus (57). In addition, a number of other preclinical animal models have demonstrated protective immune responses to bovine herpes virus (58), herpes simplex virus in rodents (14, 59–61), rabies virus (62), lymphocytic choriomeningitis virus (63, 64) and cottontail rabbit papilloma virus (65).

Allergic diseases

Allergic diseases are the result of an enhanced Th2 response to the allergens (66, 67). The deleterious response is triggered by allergen-specific IgE antibodies bound to IgE receptors at the surface of mast cells and basophils. The presence of allergen causes cross-linking of the bound IgE and results in the immediate release of histamine, IL-4 and IL-5 (68) as well as in the subsequent production of proinflammatory leukotrienes and platelet-activating factor. As allergen-encoding pDNA injected im or id into mice elicits a long-lasting antigen-specific cellular and humoral response with a Th1 phenotype (14, 15), this may provide a novel method of immunotherapy for the treatment of allergic diseases.

Cancer immunotherapy

The main idea behind immunotherapy for cancer (for review, see (69)) is based on the idea that tumour cells express unique antigens or over-express normal differentiation proteins that allow them to be recognized by the adaptive immune system as foreign. Mutations in tumour oncogenes or suppressor genes which lead to malignant transformation may also be recognized as 'foreign' tumour-specific antigens. Studies by Conry et al (70) have demonstrated the induction of a human carcinoembryonic antigen (CEA) response by the injection of pDNA encoding CEA and the subsequent protection of mice from syngeneic CEA-expressing tumour cell lines. CEA is expressed at high levels in human colon, breast and non-small-cell lung cancer. Likewise, studies by Graham et al (71) showed that pDNA encoding the polymorphic epithelial mucin (PEM) associated with breast, pancreatic and colon cancers, protected mice from challenge with syngeneic PEM-expressing tumour cells. The development of DNA vaccines which can elicit an

inflammatory response with Th1-inducing cytokines from cells of the innate immune system, along with a strong cell-mediated immune response to a specific tumour antigen, offers great potential for the future.

Advantages and disadvantages of DNA vaccines

One of the greatest advantages of genetic vaccines is the relative ease with which they can be constructed. The simplicity with which DNA can be manipulated also allows one to manipulate the antigenicity of a protein. Furthermore, more than one plasmid may be injected at a time to enable to manipulate the desired immune response to the antigen. Synthesis and purification of DNA are also rather simple compared with the conventional vaccines in which attenuated pathogens or recombinant proteins are used. DNA is also a relatively heat-stable molecule and it is especially useful in developing nations where storing vaccines in a cold environment poses an additional challenge. Furthermore, unlike protein vaccination, immunization with pDNA provides both an extended period of antigenic expression and the adjuvant effects of the immunostimulatory sequences to continuously stimulate the immune system.

Safety issues

One concern with the use of DNA vaccines is the possibility of integration into the host genome and, depending on the site of integration, the risk of affecting the expression of genes controlling cell growth, hence increasing the risk of malignancy. In addition, the strong Th1 cytokine milieu induced by DNA vaccines may actually be counterproductive in the presence of an ongoing infection in which a Th2 response could be more effective. Although DNA immunization has been clearly shown to induce potent immune responses in several animal models, it is not clear whether vaccination of widely outbred species, such as humans, will be as effective and whether the vaccination might even induce tolerance to the antigen rather than immunity in some number of individuals depending on genetic make-up or simply maturity of the immune system. DNA immunization against the circumsporozoite protein of *Plasmodium yoelii* in neonatal mice resulted in persistent tolerance (over 9 months), and in aged mice it produced significantly lower humoral and cell-mediated immunity (and provided less protection) than in young adult mice (72). However, not all DNA vaccines induce tolerance in neonates (73).

The possibility of DNA vaccines influencing the immune system (either by inducing potent antigen-specific immune responses or through the innate adjuvanticity of the DNA molecule itself) to induce responses to self-antigens and consequently trigger autoimmunity is another uncertainty. Studies by Klinman et al (72) demonstrate a threefold rise in the number of splenocytes producing anti-DNA antibodies after DNA booster immunizations compared with mice who only received primary immunizations. Despite these increases, in mice prone to spontaneous overproduction of pathogenic IgG anti-DNA antibodies vaccination did not significantly increase the production of autoantibodies to DNA or myosin. In addition, mice immunized four times with pDNA remained healthy with no signs of glomerulonephritis or myositis throughout the 16-month observation period, and kidneys from vaccinated mice showed no evidence of glomerulonephritis or immune complex deposition.

Conclusion

The relative ease of development and production as well as their efficacy in animal models make DNA vaccines an attractive mode of treatment and investigation. While not all safety concerns have been completely addressed, human trials such as the studies in HIV patients are encouraging. In summary, DNA vaccination shows promise in a number of areas including infectious diseases, allergy and cancer immunotherapies.

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Cross-Reactions between the Cytotoxic T-Lymphocyte Responses of Human Immunodeficiency Virus-Infected African and European Patients

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The great variability of protein sequences from human immunodeficiency virus (HIV) type 1 (HIV-1) isolates represents a major obstacle to the development of an effective vaccine against this virus. The surface protein (Env), which is the predominant target of neutralizing antibodies, is particularly variable. Here we examine the impact of variability among different HIV-1 subtypes (clades) on cytotoxic T-lymphocyte (CTL) activities, the other major component of the antiviral immune response. CTLs are produced not only against Env but also against other structural proteins, as well as some regulatory proteins. The genetic subtypes of HIV-1 were determined for Env and Gag from several patients infected either in France or in Africa. The cross-reactivities of the CTLs were tested with target cells expressing selected proteins from HIV-1 isolates of clade A or B or from HIV type 2 isolates. All African patients were infected with viruses belonging to clade A for Env and for Gag, except for one patient who was infected with a clade A Env-clade G Gag recombinant virus. All patients infected in France were infected with clade B viruses. The CTL responses obtained from all the African and all the French individuals tested showed frequent cross-reactions with proteins of the heterologous clade. Epitopes conserved between the viruses of clades A and B appeared especially frequent in Gag p24, Gag p18, integrase, and the central region of Nef. Cross-reactivity also existed among Gag epitopes of clades A, B, and G, as shown by the results for the patient infected with the clade A Env-clade G Gag recombinant virus. These results show that CTLs raised against viral antigens from different clades are able to cross-react, emphasizing the possibility of obtaining cross-immunizations for this part of the immune response in vaccinated individuals.

Genetic variability is one of the most remarkable hallmarks of human immunodeficiency virus (HIV), and it represents a major obstacle to the design of a vaccine against this virus. Due to this characteristic, neutralizing antibodies which are predominantly directed against the V3 loop of the envelope protein (gp120) react with only a small number of virus isolates (2, 27, 34). Other antibodies, especially those directed against conformational epitopes of the CD4 ligand of gp120 or transmembrane protein gp41, can neutralize a wider range of HIV type 1 (HIV-1) isolates (reviewed in reference 9). However, these antibodies are rarely, if ever, induced by vaccination. Cytotoxic T lymphocytes (CTLs) are thought to be another important component of the antiviral immune response. Indeed, the capacity of HIV-specific CTLs to efficiently limit viral replication is suggested by a large decrease in HIV load following the initial appearance of CTLs during primary infection (reviewed in reference 32) and by the temporal association between high CTL activity and stable viral load or $CD4^+$ cell counts during asymptomatic stages (16, 28, 29). Furthermore, HIV-exposed but seronegative individuals, as well as uninfected children born to HIV-1-infected mothers, have exhibited anti-HIV $CD8^+$ CTL reactivity as a unique sign of virus exposure (6, 31). Thus, it is generally accepted that vaccination must induce CTLs as well as neutralizing antibodies, so that infected cells can be killed before they produce any virus.

There are many target epitopes of CTLs, depending on donor HLA specificities; about 90 epitopes have been identified on the various structural and regulatory proteins of the virus (4, 13–15, 17, 18, 35, 36, 38, 41, 42; reviewed in reference 3). However, most experiments have involved lymphocytes from European or American donors infected with viruses of clade B. CTL activity has been reported for HIV type 2 (HIV-2)-infected patients (1, 12, 26, 31), but to our knowledge only one study has concerned African people infected with African HIV-1 isolates (31). We studied lymphoid cells from the blood of clade A virus-infected African patients and/or clade B virus-infected French patients. Both were tested against autologous target cells infected with recombinant viruses expressing various proteins from clade A or B viruses or from HIV-2. The large degree of cross-reactivities observed suggests that the variability of viral proteins will not be an obstacle in obtaining cross-reacting CTL in vaccinated individuals.

MATERIALS AND METHODS

Subjects. Heparinized blood samples were collected from 16 consenting HIV-1-seropositive individuals, 7 in Bangui (Central African Republic) and 9 in France (1 was originally from Togo; patient W121). They were first diagnosed as HIV positive between 1989 and 1995. All had circulating anti-HIV-1 antibodies but not anti-HIV-2 antibodies. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and frozen. HLA-A, -B, and -C types were determined serologically by the Laboratory of Immunology and Histocompatibility at Hospital Saint-Louis, Paris, France: B12 (HLA-A3/32, B41/–, C3/6); B15 (HLA-A3/31, B7/52, C6/–); B16 (HLA-A23/24, B13/47, C2/–); B18 (HLA-A2/31, B13/55, C2/6); B20 (HLA-A2/30, B7/13, C3/–); B22 (HLA-A2/30,

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B27/44, C2/−); B23 (HLA-A19.2/−, B44/57, C6/−); and W121 (HLA-A2/33, B50/70, C2/6).

Genetic subtyping of HIV-1 strains. The genetic subtypes of HIV-1 were determined by a heteroduplex mobility assay (8). The C2V5 region of the *env* gene was amplified by nested PCR with the ED5 and ED12 primers for the first round and the ES8 primers for the second round. The *gag* gene was amplified by nested PCR with the G00 and G01 primers for the first round and the G60 and G25 primers for the nested PCR (33). The amplified fragment was then purified with a PCR product purification kit (33) and sequenced with a p24-specific internal primer by using dye terminator chemistry (Perkin-Elmer) on an automated DNA sequencer (Applied Biosystems 373A). DNA sequences were analyzed with the multiple sequence analysis program CLUSTALW (37). Reference strains for each subtype for the region analyzed were included in this study. Tree topology based on 634 nucleotides was inferred by the neighbor-joining method.

Vaccinia viruses. The viruses used to infect target cells were vaccinia viruses (Copenhagen strain) recombined with the complete *env*, *gag*, *pol*, or *nef* gene of HIV-1 (LAI strain) (resulting in viruses Env/LAI, Gag/LAI, Pol/LAI, and Nef/LAI). Recombinant viruses encoding various proteins, such as gp120, gp41, p24, p18, reverse transcriptase (RT), integrase, or protease, were also used. They were produced as previously described (20, 21). An initiation codon, a stop codon, and adequate restriction sites were introduced during the construction of the Nef-1 (codons 1 to 72), Nef-2 (codons 73 to 147), and Nef-3 (codons 145 to 206) regions of Nef by local mutagenesis immediately before and after the indicated positions. Other recombinant vaccinia viruses were also constructed to express genes from isolates of subtype A of HIV-1 or of HIV-2. Gag/CAR, Pol/CAR, and Nef/CAR vaccinia viruses expressed the corresponding genes from HIV-1 92CAR3253 (obtained from a Central African Republic patient). The corresponding DNA fragments were amplified by PCR from DNA extracted from human PBMC infected with this isolate (obtained from F. Barré-Sinoussi). Adequate restriction sites were introduced during the amplification procedure. Pol/CAR expressed the complete *pol* gene. The Gag protein produced by Gag/CAR lacked 36 amino acids at the C terminus and ended with PPAEI. The Nef protein produced by Nef/CAR lacked 3 amino acids at the C terminus and ended with MKPEF. Env/OUG vaccinia virus expressed the native envelope protein gene of HIV-1 92UG037 (from a Ugandan patient). Env/ROD, Gag/ROD, and Nef/ROD vaccinia viruses expressed the corresponding genes from HIV-2 (ROD strain) (obtained from a West African patient). These genes were excised from the genome of HIV-2_{ROD} and subjected to local mutagenesis to introduce restriction sites before the ATG initiation codon and after the stop codon. They were then introduced into the genome of the vaccinia virus. The stop codon in the gp36 coding sequence was removed by local mutagenesis to restore the reading frame of the native envelope protein gene.

Peptides. Peptides corresponding to epitopes previously identified in clade B viral sequences (23) were synthetized by Neosystem (Strasbourg, France): Gag 77-85 (SLYNTVATL) (39), Gag 263-272 (KRWIILGNK) (25), Nef 73-82 (QV PLRPMTYK) (19), and Nef 136-145 (PLTFGWCFKL) (15). They were supplied by the Agence Nationale de Recherche sur le SIDA. Lyophilized peptides were dissolved in water (2 mg/ml) and stored at −20°C.

Generation of anti-HIV cell lines. Polyclonal anti-HIV cell lines were obtained by culturing PBMC (10^6/ml) with autologous phytohemagglutin-activated lymphocytes (2×10^5/ml) as described previously (20). The cells were incubated for 3 days in RPMI 1640 (GIBCO) supplemented with 2 mM L-glutamine, 10 mM HEPES buffer, and 10% fetal calf serum. They were then cultured at a concentration of 10^6/ml in medium supplemented with 10 U of human recombinant interleukin 2 (Boehringer) per ml. Cytolytic activity was tested after 14 to 21 days in culture.

Antipeptide cell lines were generated in some experiments by use of the same culture medium as that described above and by coculturing 10^7 PBMC (4×10^6/ml) with a similar number of autologous PBMC which had been treated with a pulse of 1 μg of peptide for 90 min and irradiated. Continuous cell lines were established by similar weekly stimulation as previously described (7).

CRT. The target cells used in the chromium release test (CRT) were autologous lymphoblastoid cells obtained by transforming PBMC with Epstein-Barr virus (EBV-LCL). They were infected with recombinant vaccinia viruses by incubation with 5 PFU per cell for 18 h. Wild-type vaccinia virus (Vac/WT) was used as a control. EBV-LCL were labeled by incubation with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham) for 1 h and washed twice. EBV-LCL incubated with 1 μg of peptide for 90 min and then extensively washed were used as target cells in some experiments. The control consisted of target cells incubated with medium alone.

The CRT was performed with microculture plates by incubating various concentrations of effector cells and 5×10^3 target cells in RPMI 1640 supplemented with 10% fetal calf serum for 4 h. The supernatants were then harvested, and the chromium released was measured in a gamma counter. The spontaneous release was 10 to 25% of the total Cr incorporated. The specific chromium release was calculated as 100 × [(experimental − spontaneous release)/(total Cr incorporated − spontaneous release)]. HIV-specific activity was considered to be present when the specific chromium release was 10% greater than that of the control Vac/WT for two different effector/target cell ratios. Lytic units (LU) were calculated for 10^8 effector cells as $10^8/(5{,}000 \times E/T^{30\%})$, where $E/T^{30\%}$ is the effector/target cell ratio that yields 30% specific lysis of 5,000 target cells. LU for Vac/WT were always less than 3.

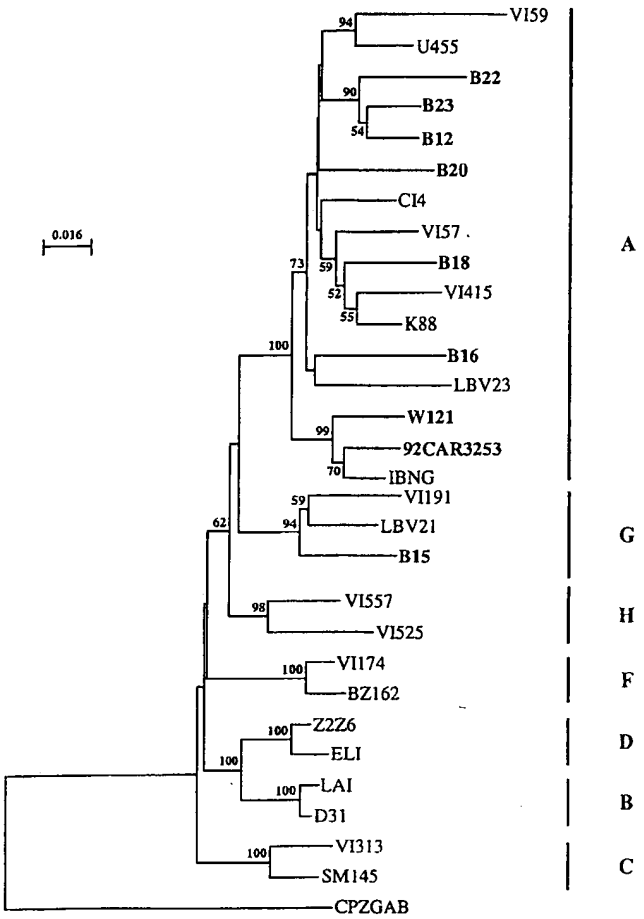

FIG. 1. Phylogenetic analysis of *gag* nucleotide sequences. The phylogenetic tree was generated by the neighbor-joining method and drawn with Njplot. The numbers given at the branch points are the 50% threshold majority consensus values for 100 bootstrap replicates. The lengths of the horizontal branches are proportional to the relative evolutionary distances; vertical distances are for clarity only. The strains isolated from the African patients described in this study, as well as the strain used to produce the recombinant vaccinia viruses encoding the *gag* gene (92CAR3252), are shown in bold type.

Nucleotide sequence accession numbers. The nucleotide sequences of the HIV-1 *gag* p24 region for patients B12, B15, B16, B18, B20, B22, B24, and W121 were deposited in the EMBL Nucleotide Sequence Database under accession no. Y16612 to Y16619, respectively.

RESULTS

Genetic subtyping of HIV-1 strains. The samples from the Caucasian patients all formed fast-migrating heteroduplexes with the subtype B reference strain. The viruses originating from Bangui (B12, B15, B16, B18, B20, B22, and B23) all clearly formed fast-migrating heteroduplexes with the subtype A reference strain, as did the viral isolate from the patient from Togo (W121). Many different genetic subtypes have been found in the Central African Republic (24, 33a): A, E, D, C, H, G, and U (decreasing order of frequency); it is possible that some of the isolates studied were indeed recombinant genomes (11, 30). However, as no subtype B was found in this country, it is unlikely that any of the Bangui isolates were A-B recombinants. We sequenced part of the Gag region for Bangui isolates and for the virus from Togo. Seven of the eight viruses were identified as belonging to clade A (Fig. 1). One virus

TABLE 1. CTL activities in patients infected with subtype A HIV-1

Target antigen	LU[a] for donor:							
	B12	B15	B16	B18	B20	B22	B23	W121
Subtype A HIV-1								
Env/OUG	<3	<3	190	277	<3	<3	<3	233
Gag/CAR	<3	33	85	65	99	95	97	25
Pol/CAR	92	47	583	62	61	52	101	75
Nef/CAR	73	24	285	116	<3	63	98	30
Subtype B HIV-1								
Env/LAI	<3	<3	<3	80	<3	<3	<3	<3
Gag/LAI	<3	27	65	58	64	42	61	130
Pol/LAI	79	32	143	28	36	21	<3	17
Nef/LAI	47	<3	234	93	<3	33	<3	23
HIV-2								
Env/ROD	<3	<3	<3	<3	<3	<3	<3	<3
Gag/ROD	<3	26	<3	37	74	<3	<3	<3
Nef/ROD	<3	<3	<3	<3	<3	<3	<3	<3

[a] LU were calculated as described in Materials and Methods.

(B15) clustered with the subtype G isolates, showing that this virus was an A-G recombinant.

Reactivity of CTLs stimulated with endogenous virus. Lymphoid cells from the 16 patients were stimulated in vitro with autologous phytohemagglutinin-activated blast cells, so that the restimulating viral proteins were from endogenous viruses from the same patient. CTL reactivities were tested against a panel of structural proteins (Env, Gag, or Pol) from clade A or B viruses and against Nef (from clade A and B viruses), as Nef is the most frequently recognized regulatory protein (20). Reactivities with the Env, Gag, and Nef proteins of HIV-2_{ROD} were also tested. The reagent for testing HIV-2 Pol was not available.

The results found with CTLs from clade A virus-infected patients are summarized in Table 1, and an example is shown in Fig. 2A. The CTLs from these eight patients clearly reacted with several clade A proteins; CTLs from three of them reacted with all four proteins tested, CTLs from three reacted with three proteins, and CTLs from two reacted with two proteins. Pol epitopes were recognized by CTLs from all donors, Gag and Nef epitopes were recognized by CTLs from most of them (seven of eight), but Env-reacting CTLs were found in only three of the eight donors. Figure 2A shows that B18-derived CTLs recognized equally well the Gag epitopes of CAR (clade A), LAI (clade B), and even ROD (HIV-2) viruses. Clear cross-reactivities were also found with Env, Pol, and Nef; in all cases, the levels of the responses were equivalent for CAR or LAI viruses. In contrast, cross-reactivities against ROD (HIV-2) were found only for Gag. CTLs from the other seven African patients also showed multiple cross-reactions with proteins of clade B viruses. The levels of their responses to Gag proteins of the two subtypes were similar. The reactivity with Pol was stronger with the homologous protein. Nevertheless, CTLs from all but one donor reacted with Pol/LAI. Finally, five of the seven donors had CTLs that recognized Nef/CAR and Nef/LAI in the same response range. In contrast, cross-reactivity with the Env protein was weaker; only one of the three donors (B18) whose CTLs reacted with Env/CAR had CTLs that also reacted with Env/LAI, but with weaker activity. CTLs from clade A-infected donors seldom cross-reacted with HIV-2 proteins. No cross-reactivity was found with Env or Nef, and reactivities against Gag/ROD were observed with CTLs from only three donors among the eight whose CTLs were capable of recognizing Gag/CAR.

Similar experiments with lymphoid cells from clade B-infected European patients yielded similar results (Table 2). CTLs from all eight donors reacted with several proteins from clade B viruses, consistent with previous work (20). Figure 2B shows the strong cross-reactivities of CTLs from the donor carrying virus CO3M with Env, Gag, and Nef proteins of clades A and B. Altogether, the CTLs of most clade B-infected patients (seven of eight) reacted with Nef/CAR (clade A), and the response was in the same range as that for Nef/LAI. Cross-reactivities against Env/OUG (four of eight), Gag/CAR (five of eight), and Pol/CAR (six of eight) were also found. Finally, no CTLs from any of the clade B-infected patients were found to react with target cells expressing ROD (HIV-2) Env, Gag, or Nef.

Cross-reactivities of CTLs with various Env, Gag, Pol, and Nef subregions. It is important for vaccine development to further determine the precise targets of cross-reacting CTLs. To do this, we used target cells infected with recombinant vaccinia viruses expressing Env gp120, Env gp41, Gag p24, Gag p18 (the recombinant for Gag p15 was not available), RT, integrase, or protease. Three recombinants expressing the N-terminal (Nef-1), central (Nef-2), or C-terminal (Nef-3) regions of Nef were also tested. All were from the clade B LAI isolate, the corresponding clade A reagents not being available. Previous experiments largely documented the capability of CTLs from patients infected with clade B viruses to react against these different proteins (reviewed in reference 40). This characteristic was tested with CTLs from African patients (Table 3). Reactivity against Env was rare, found in only one of the seven donors (B18), who produced CTLs specific for both gp120 and gp41 of the LAI isolate. On the contrary, broad cross-reactivities were detected with Pol and Gag, as p24 and p18 from the LAI isolate were recognized by CTLs from four of the six clade A-infected donors; RT was recognized by CTLs from two of the seven, protease was recognized by CTLs from only one of the seven, and remarkably enough integrase was recognized by CTLs from all seven of the African donors (at least four of them had a strong response). Finally, the reactivities detected against Nef in five of the seven patients always revealed cross-reacting epitopes in the central region of this protein.

Identification of conserved epitopes. We investigated the presence in African patients of CTLs specific for peptides previously identified as epitopes in clade B viruses. This was feasible, since all of these donors had at least one class I molecule corresponding to a known epitope already studied in European or American patients. When PBMC were still available, anti-peptide cell lines were produced, and CTL activity was tested against target cells sensitized with the corresponding peptide or infected with recombinant vaccinia virus. It is important to note that this experimental approach allows the detection of CTLs from PBMC of only infected (or vaccinated) individuals.

CTLs from patient B18 recognized epitopes Gag 77-85 (39) and Nef 136-145 (15), which are known to be HLA-A2 restricted (Fig. 3a and b). These CTLs also lysed target cells infected with recombinant vaccinia viruses carrying the corresponding genes from the LAI and CAR isolates but not the HIV-2_{ROD} isolate. Similarly, epitope Nef 73-82, which is restricted to HLA-A3 (19), was recognized by CTLs from patient B12 (Fig. 3c). Finally, CTLs from patient B22 reacted with the well-known HLA-B27-restricted epitope Gag 263-272 (25) (Fig. 3d) but not with the HLA-A2-restricted epitope Nef 136-145.

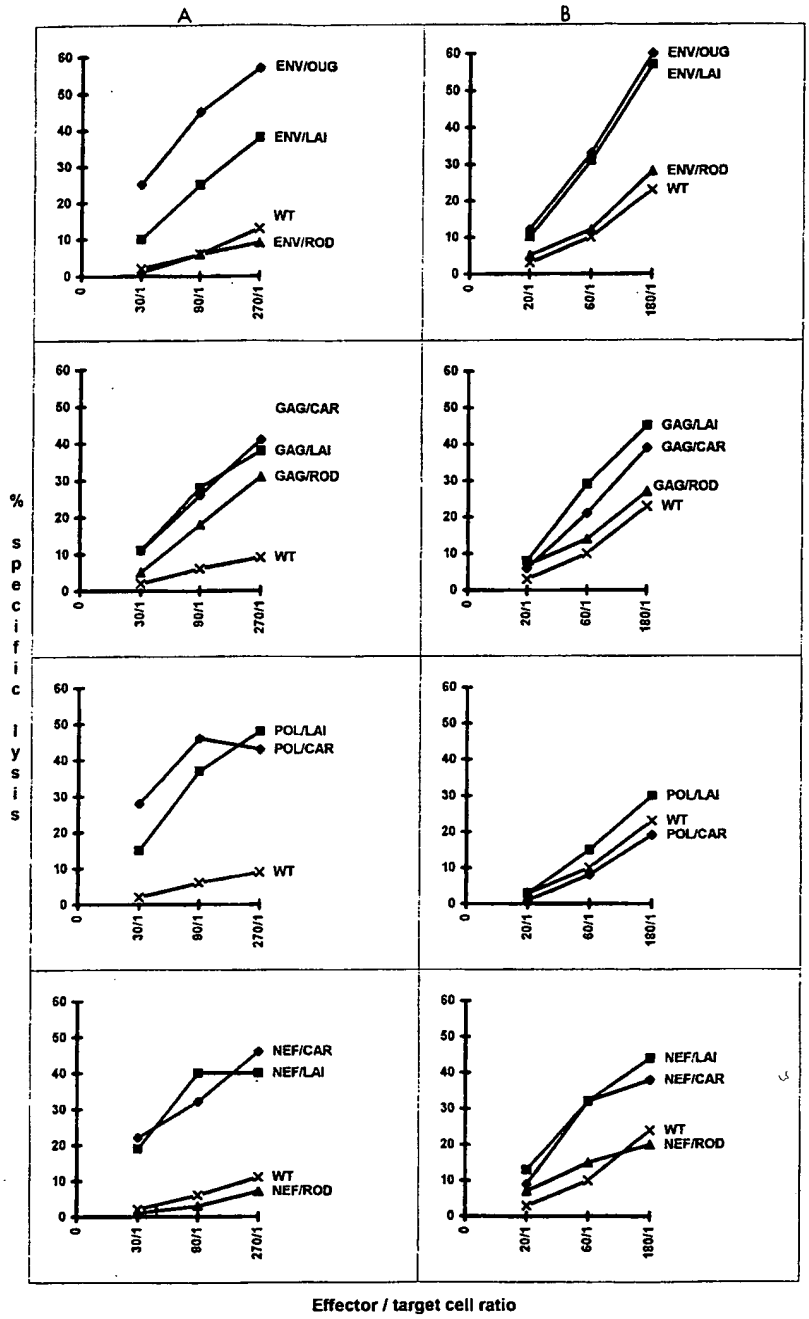

FIG. 2. CTL activities in patients carrying viruses B18 (infected with clade A HIV-1) (A) and CO3M (infected with clade B HIV-1) (B). Effector cells were tested after in vitro restimulation with autologous blast cells. Target cells were infected with recombinant vaccinia viruses expressing the Env, Gag, Pol, or Nef protein of clade A and B isolates.

DISCUSSION

Anti-HIV-1 CTL responses have been studied almost exclusively in Europe and North America with lymphoid cells from people infected with clade B viruses. They are directed against several proteins of the same virus and often against several epitopes of the same protein. This polymorphism of CTL responses is well documented for these viruses (reviewed in reference 40). Our results show that the same polymorphism exists for anti-clade A virus CTL responses (Table 1). CTLs from all eight patients tested responded to several structural proteins and also frequently to Nef, with at least three to five different CTL activities in the same donor (Table 3). It is interesting to note, however, that Env was recognized by the CTLs from only three of the eight patients, suggesting a low prevalence of broadly cross-reactive Env-specific CTLs among clade A virus-infected individuals, consistent with results reported for clade B viruses (5). Moreover, this study clearly demonstrates frequent cross-reactivities in the CTL responses obtained after infection with viruses belonging to two different clades. Several patients had almost identical CTL reactions to Gag and Nef proteins from clade A and B viruses, regardless of whether the original virus was of clade A or B. The homolo-

TABLE 2. CTL activities in patients infected with subtype B HIV-1

Target antigen	LU[a] for donor:							
	CO3M	T051	BI02	MIO7	MI10	MI21	W39	W44
Subtype B HIV-1								
Env/LAI	143	<3	87	18	83	134	28	486
Gag/LAI	77	28	178	84	108	507	57	567
Pol/LAI	<3	77	75	159	77	312	37	128
Nef/LAI	93	34	127	72	23	219	61	<3
Subtype A HIV-1								
Env/OUG	125	<3	<3	<3	18	<3	62	42
Gag/CAR	38	<3	<3	43	47	<3	59	56
Pol/CAR	<3	42	45	83	23	<3	17	103
Nef/CAR	73	18	96	28	12	49	23	<3
HIV-2								
Env/ROD	<3	<3	<3	<3	<3	<3	<3	<3
Gag/ROD	<3	<3	<3	<3	<3	<3	NT	<3
Nef/ROD	<3	<3	<3	<3	<3	<3	<3	<3

[a] LU were calculated as described in Materials and Methods. NT, not tested.

TABLE 3. CTL activities against various proteins of HIV-1_{LAI}

Target antigen	LU[a] for donor:						
	B12	B15	B16	B18	B20	B22	W121
Env/LAI	<3	<3	<3	80	<3	<3	<3
Gp120	<3	<3	NT	37	NT	<3	<3
Gp41	<3	<3	NT	28	NT	<3	<3
Gag/LAI	<3	27	65	58	64	42	130
P24	NT	14	<3	12	<3	33	110
P18	NT	19	<3	35	51	<3	68
Pol/LAI	79	32	143	28	36	52	17
RT	60	<3	<3	43	<3	<3	<3
Int	43	41	179	56	18	37	26
Pro	<3	<3	<3	<3	12	<3	<3
Nef/LAI	47	<3	234	93	<3	63	23
Nef-1	<3	<3	NT	<3	<3	<3	<3
Nef-2	38	<3	56	82	<3	82	42
Nef-3	<3	<3	NT	<3	<3	<3	<3

[a] LU were calculated as described in Materials and Methods. NT, not tested.

gous proteins sometimes elicited a stronger response, notably with Pol targets, but even in this case cross-reactivity was evident. Weaker cross-reactivities were detected against Env proteins. The CTLs of African patients were selective for homologous clade A Env. CTLs from only one donor showed a cross-reaction with Env/LAI. In the reverse situation, CTLs from four of the eight European donors cross-reacted with clade A Env. It is not surprising that Gag, Pol, and Nef were better targets for cross-reaction than Env, since the variability of Env is especially important and well known.

It must be emphasized that the classical methods used to test CTLs allow demonstration only of responses directed to conserved epitopes, as patients are stimulated with epitopes of their own viruses, which are variable, while the tests are carried out with target cells infected with a single recombinant vaccinia

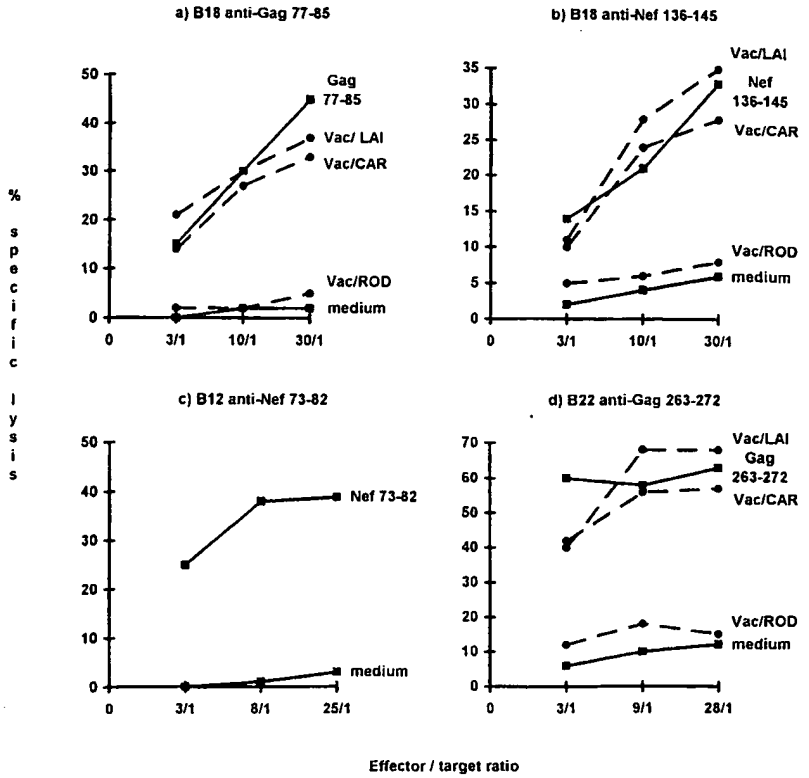

FIG. 3. Recognition of known epitopes by CTLs from African patients. CTLs were produced by in vitro stimulation with synthetic peptides. They were tested against target cells previously treated with the corresponding peptide or infected with recombinant vaccinia virus (Vac) (except for the B12 cell line, because effector cells were not available). The HLA typing was as follows: for B12, HLA-A3/32, B41/−, C3/6; for B22, HLA-A2/30, B27/44, C2/−; and for B18, HLA-A2/31, B13/55, C2/6. The target cells were heterologous EBV-LCL sharing HLA-A3 with B12 CTLs, HLA-B27 with B22 CTLs, or HLA-A2 with B18 CTLs.

virus. The target cells for clade B viruses generally express viral proteins from HIV-1_{LAI}, so only epitopes conserved between LAI and the infecting virus can be revealed in the reaction. Similarly, the recently produced panel of recombinants expressing different clade A proteins was produced with a single clade A virus, so only epitopes conserved among clade A virus-infected donors can be detected. The same epitopes are probably responsible for most of the cross-reactivity between the two clades, as few differences were detected in cross-reactions involving Gag, Pol, or Nef. The target epitopes map to Gag p18 and p24 and the central region of Nef, as has already been shown for clade B viruses (3). Our results suggest a particular importance for integrase epitopes with constant cross-reactivities between clade A and B viruses (Table 3). Reactions directed against integrase are poorly documented. However, we previously identified this enzyme as a good CTL target for clade B viruses (21). Cross-reactivities with HIV-2 are only occasional. We found that anti-clade A virus CTLs sometimes cross-reacted with HIV-2 Gag protein, as previously reported (12, 26, 31), whereas they did not cross-react with other proteins. On the other hand, CTLs from patients infected with clade B viruses did not cross-react with HIV-2 proteins.

It is very probable that only some of the epitopes recognized on homologous proteins are responsible for cross-reactivity. However, our results suggest that obstacles to vaccination because of the induction of broadly cross-reactive neutralizing antibodies seem not to affect CTL responses so extensively. This idea is not surprising because the target proteins of CTLs are less variable than the V3 loop and because the African patients tested shared with northern populations at least one HLA specificity capable of presenting previously identified epitopes, although we did not select for this. Further studies are required to investigate cross-reactivities with viruses of other clades, including C, D, E, and G, and even viruses of type 0. It is likely that strong cross-reactivities will be found, as one of the donors bore a virus (B15) belonging to clade G (for the Gag proteins) but reacting with clade A and B Gag proteins, including p24 and p18. Viruses of clade E also carry a clade A *gag* gene (11), so cross-reactions probably will be identified at least against Gag.

Results obtained with synthetic epitopes showed that African patients have CTL precursors which react with previously identified epitopes in Gag and Nef proteins of clade B isolates. Similar experiments performed with European patients allowed us to discover such cross-reactivities at the epitope level (data not shown). For example, CTLs specific for the Nef 84-92 epitope (7) were able to recognize target cells infected with Vac/CAR. This result is not surprising, since the peptide sequence is conserved between LAI and CAR isolates. A similar finding was obtained for the RT 325-333 epitope (41), suggesting that the mutation Ser (LAI isolate) → Ala (CAR isolate) at position 8 did not affect the epitope. Similarly, mutations observed in CAR isolates at the level of the Nef 73-82, Gag 77-85, and Gag 263-272 epitopes did not induce escape of recognition by CTLs from African patients (Fig. 3). These results are consistent with previous reports of conserved epitopes shown with CTLs from patients infected with clade B isolates (reviewed in reference 22) or from uninfected vaccinated volunteers (10). They show that cross-reactivity can be due to conservation of epitope sequences as well as to cross-recognition of epitopes which differ in amino acid sequences. However, further studies are necessary to identify the precise epitopes involved in cross-reactions. This identification may be of value in developing a vaccination system based on peptides or lipopeptides, although the level of CTL response required for possible in vivo protection is not known.

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IMMUNOGENIC CONJUGATES

This application is a continuation in part of application Ser. No. 298,102, filed Aug. 31, 1981, now abandoned, which is incorporated herein by references.

TABLE OF CONTENTS

1. FIELD OF THE INVENTION

This invention relates to the field of novel vaccine compositions, processes for producing them and methods for immunization of young warm-blooded animals, including humans, against infections and disease caused by bacteria, including, for example, *Haemophilus influenzae* type b, *Escherichia coli, Neisseria meningitidis* serogroups A and C, *Streptococcus pneumoniae serotypes* 3, 6, 12, 14, 19, 23 and 51, and *Pseudomonas.*

2. BACKGROUND OF THE INVENTION

It is known that purified bacterial capsular polymers (CP) generally are immunogenic in mature humans and animals and can be used as vaccines against the corresponding systemic infections. As used in this application, the term "capsular polymers" refers to sugar-containing polymers, such as polymers of sugars, sugar acids, amino sugars, polyhydric alcohols and sugar phosphates, and does not refer to amino acid-containing polymers. These "capsular polymers" are frequently referred to in the medical literature as "capsular polysaccharides", though they may contain linkages other than glycosidic linkages and constituents other than sugars such as those listed above.

The capsular polymers of different bacteria vary widely in immunogenicity in the first year of human life. Some are moderately active, such as *Streptococcus pneumoniae* serotype 3 and *Neisseria meningitidis* serogroup A. The susceptibility to systemic infection by encapsulated bacteria is greater in the first year of life. The immunogenic response to many bacterial capsular polymers in children is age dependent, i.e., immunocompetence to CP increases to adult levels by about six years of age.

Among the inactive CP are those of *Haemophilus influenzae* type b, *Streptococcus pneumoniae* serotypes 6 and 12, and *Neisseria meningitidis* serogroup C. Examples of CP's which give an intermediate response in

infants are *Streptococcus pneumoniae* serotypes 19 and 51.

2.1. INTACT CAPSULAR POLYMERS AS ANTIGENS IN VACCINES

Various investigators have isolated and purified intact capsular polymers which may be useful in or as vaccines. For example, U.S. Pat. No. 4,220,717 describes a process for the isolation and purification of immunologically active polyribosyl ribitol phosphate (PRP) from the capsular polymer of *H. influenzae* b. Additionally, U.S. Pat. No. 4,210,641 relates to polysaccharide extracts of *H. influenzae* having an apparent molecular weight greater than 200,000 daltons and composed principally of galactose, glucose and mannose and containing a small amount of osamines.

Several researchers have utilized these and other intact capsular polymers in formulations to achieve better immunological responses. For example, U.S. Pat. No. 4,196,192 discloses a vaccine containing purified intact PRP and whole *Bordetella pertussis* bacteria. This approach to increasing immunogenicity resulted in enhanced levels of anti-PRP and anti-pertussis antibodies in young mammals.

2.2. VACCINES CONTAINING CONJUGATES

Other researchers have studied conjugation of capsular polymers to carrier proteins in an effort to enhance antibody formation by the so-called "carrier effect". For example, Schneerson et al., Journal of Experimental Medicine 152:361-376 (1980) describes *H. influenzae* b polymer-protein conjugates disclosed to confer immunity to invasive diseases caused by *H. influenzae* b. The reference documents the age-related immunological behavior of capsular polymers in infants and seeks to overcome this age-dependence by conjugation of the intact capsular polymer with a variety of proteins, including serum albumins, *Limulus polyphemus hemocyanin* and diphtheria toxin. The method of conjugation involves the use of a linking agent such as adipic dihydrazide.

Geyer et al., Med. Microbiol. Immunol. 165:171-288 (1979), prepared conjugates of certain *Klebsiella pneumoniae* capsular polysaccharide fragments to a nitrophenyl-ethylamine linker by reductive amination, and the derivatized sugar was then attached to proteins using azo coupling.

3. SUMMARY OF INVENTION

The present invention relates to the covalent attachment of capsular polymer fragments derived from bacterial capsular polymers to bacterial toxins or toxoids by means of reductive amination. As used in the present application, the term "toxoid" means a form of a toxin which has the antigenicity of the toxin without its toxicity.

The immunogenic conjugates of the invention are prepared by first forming reducing end groups on fragments of the capsular polymers and reacting these with amine groups of the bacterial toxin or toxoid by reductive amination. The reducing end groups may be formed by any suitable method, including selective hydrolysis, e.g., by acids or enzymes, or by oxidative cleavage, e.g., by periodate. The conjugation is preferably achieved by reductive amination in an aqueous solution containing cyanoborohydride anions.

The immunogenic conjugates of the invention may be formulated with a pharmaceutically acceptable carrier

to produce a vaccine which elicits effective levels of anti-capsular antibody formations in young mammals, including humans. The vaccine may be utilized to induce active immunization against systemic infection in young mammals caused by the respective encapsulated bacteria by administering an immunogenic amount of the conjugate to the mammal.

The immunogenic conjugates have been found to be less age dependent than the capsular polymers alone, and are useful for the active immunization of very young warm-blooded mammals against systemic infections by the respective encapuslated bacteria.

Furthermore, the immunogenic conjugates of the invention do not contain potentially toxic linking agents, such as adipic dihydrazide or p-nitro-phenylethylamine, which have been used in conjugating carbohydrate to protein.

Finally, the immunogenic conjugates of the invention contain fragments of capsular polymers, not intact capsular polymers. The highly repetitive structure of capsular polymers may be in part responsible for their failure to expand the capacity for antibody production in infants. A conjugate of intact (highly polymerized) CP and protein may only partially overcome the immunologic disadvantages of CP alone.

On the other hand, the use of capsular polymer fragments on a carrier may circumvent the disadvantages of the repetitive structure. Additionally, the CP determinants of a conjugate having CP fragments are on the average closer to the carrier than are the CP determinants of conjugates having intact CP, and this proximity to carrier may be necessary for a more effective "carrier effect".

A further advantage lies in the use, for the protein carrier, of a bacterial toxin or toxoid against which children are routinely vaccinated, e.g., tetanus or diphtheria. Desired immunity to the toxin or toxoid is induced along with immunity against the pathogens associated with the capsular polymer.

4. DETAILED DESCRIPTION OF THE INVENTION

The conjugates of the invention are formed by reacting reducing end groups of the capsular polymer fragment to primary amino groups of a bacterial toxin or toxoid to yield antigenic determinants of the capsular polymer covalently linked to the carrier protein. The reducing groups may be formed by selective hydrolysis or specific oxidative cleavage.

Antigenic fragments with at least one reducing end can be generated from capsular polymers by a variety of methods, depending upon the structural features of the particular capsular polymer. Limited oxidative cleavage by periodate (or related reagents) will leave aldehydic termini; such an approach will be limited to polymers having vicinal dihydroxy groups on a non-cyclic residue. Hydrolysis of a glycosidic linkage produces a reducing sugar terminus. Such hydrolysis can be most specifically accomplished enzymatically by glycosidases, but this application would be restricted to a relatively few capsular polymers, e.g., *Streptococcus pneumoniae* 8, for which glycosidases are known. Acidic hydrolysis is commonly used for hydrolysis of glycosidic linkages. The utility of this approach would be limited if the polymer contains acid-sensitive non-glycosidic linkages or if the polymer contains acid-sensitive branch linkages important to the antigenic specificity.

The conjugation is carried out according to the reductive amination process of Schwartz and Gray, Arch. Biochem. Biophys. 181:542-549 (1977). Briefly, the process involves reacting the reducing capsular polymer fragment and bacterial toxin or toxoid in the presence of cyanoborohydride ions, or another reducing agent which will not reduce the reducing ends of interest nor adversely affect the toxin or toxoid capsular polymer. The cyanoborohydrate ions (or their equivalent) act solely as a mild selective reducing agent of the Schiff base intermediate formed between the carbonyl groups of the hydrolyzed capsular polymer fragment and amino groups of the protein. Thus, unlike previously employed conjugation procedures wherein the active molecules are joined by a linking agent which forms a part of the final product, the cyanoborohydride reducing anions utilized herein are not incorporated into the final product. This is important from the standpoint of controlling the potential toxicity of the final product. Evidence of covalent linkage is demonstrated by the fact that the association between, for example, a PRP moiety and the carrier protein persists despite salting-out of the protein in the presence of 8M urea, which has a great ability to disrupt non-covalent bonds.

Suitable carrier proteins are those which are safe for administration to young mammals and immunologically effective as carriers. Safety would include absence of primary toxicity and minimal risk of allergic complications. Diphtheria and tetanus toxoids fulfil these criteria; that is, suitably prepared, they are non-toxic and the incidence of allergic reactions is well documented. Though the risk of allergic reaction may be relatively significant for adults, it is minimal for infants.

In the "carrier effect" a weak antigen, by being attached to a stronger antigen as carrier (i.e., a heterologous protein), becomes more immunogenic than if it were presented alone. If an animal is previously immunized with the carrier alone, it may become "primed" for an enhanced response not only to the carrier antigen but also the attached weaker antigen. Infants are routinely immunized with tetanus and diphtheria toxoids. Thus, they would be primed for subsequent presentation of a capsular polymer antigen conjugated to either of these toxoids.

In general, any heterologous protein could serve as a carrier antigen. However, certain bacterial toxins such as tetanus and diphtheria may have an additional advantage in that they are composed of two portions, one of which (the "binding" subunit) has a strong affinity for binding to mammalian cell surfaces. Conceivably, conjugation to such a "binding" protein would permit the carried antigen to more effectively initiate responses in cells of the immune system.

The carrier proteins to which the capsular polymer is conjugated may be native toxin or detoxified toxin (toxoid). Also, by relatively recent mutational techniques, one may produce genetically altered proteins which are antigenically similar to the toxin yet non-toxic. These are called "cross reacting materials", or CRMs. CRM_{197} is noteworthy since it has a single amino acid change from the native diphtheria toxin and is immunologically indistinguishable from it.

A culture of *Corynebacterium diphtheriae* strain C7 (197), which produces CRM_{197} protein, has been deposited with the American Type Culture Collection, Rockville, Maryland and has been assigned accession number ATCC 53281.

Conjugation of capsular polymer to native toxin may reduce toxicity, but significant toxicity may remain. Thus, further detoxification would be required. Conventional detoxification of protein toxins employs formalin, which reacts with free amino groups of the protein. Residual toxicity may still be a concern. Furthermore, spontaneous retoxification is possible with any particular lot of vaccine and remains an issue of concern with this approach.

Alternatively, native toxin may be detoxified with formalin to produce conventional toxoid before conjugation to capsular polymer. However, the prior formalin treatment reduces the number of free amino groups available for reaction with the reducing groups of the capsular polymer fragment. CRMs, thus, have significant advantages in that they have no inherent toxicity yet none of their amino groups are occupied by the formalin. A further advantage is that no biohazards exist in working with CRMs.

In the case of CRM_{197}, which is immunologically identical to native toxin, treatment with formalin (though there is no need to detoxify) greatly enhances the immunological response. It is thought that this is due to stabilization of the molecule against degradation by mechanisms of the body and/or aggregation by cross-linking (immunogenicity of particles increases with size).

For all of the above reasons, tetanus and diphtheria toxins are prime candidates for carrier proteins, yet there are others which may also be suitable. Though these others may not have the history of safety found with diphtheria and tetanus, there may be other overwhelming reasons to use them. For instance, they may be even more effective as carriers, or production economics may be significant. Other candidates for carriers include toxins of pseudomonas, staphylococcus, streptococcus, pertussis and *Escherichia coli.*

Suitable carrier media for formulating a vaccine include sodium phosphate-buffered saline (pH 7.4) or 0.125M aluminum phosphate gel suspended in sodium phosphate-buffered saline at pH 6 and other conventional media.

Generally, vaccines containing from about 5 to about 100 μg, preferably about 10 to 50 μg, are suitable to elicit effective levels of antibody against the capsular polymer in young warm-blooded mammals. Of course, the exact dosage would be determined by routine dose/response experimentation. Several small doses given sequentially would be expected to be superior to the same amount of conjugate given as a single injection.

The vaccines of the invention may be administered by injection to warm-blooded mammals of any age and is especially adapted to induce active immunization against systemic infections in young mammals caused by the pathogens *Haemophilus influenzae* type b, *Escherichia coli,* pneumococcus, meningococcus, streptococcus and pseudomonas.

The following are non-limiting examples of methods for the preparation of exemplary immunogenic conjugates of the present invention and their use in vaccines.

5. EXAMPLE: GENERATION OF LARGE, MEDIUM AND SMALL FRAGMENTS OF PRP CONTAINING REDUCING END GROUPS AND CONJUGATION TO CRM_{197}

The capsular polymer of *Hemophilus influenzae* type b is a linear polymer with the repeating unit [-3-β-D-ribosyl(1-1)ribitol(5-phosphate)-] (PRP). Generally, hydrolysis of PRP is carried out until the ratio of total to reducing ribose has dropped to 25 or below. The resulting mixture of size fragments may be fractionated by molecular sieve column chromatography to isolate the desired size range of fragments for conjugations. The method for obtaining fragments is as follows:

a. A sample of sodium PRP, (nucleic acid content 0.006%) containing 28.6 milligrams ribose was dissolved with distilled water to make a total volume of 9.2 ml in a 125-ml erlenmeyer flask and chilled in ice.
b. 1.02 ml of $0.1N_2SO_4$ was added.
c. Duplicate samples of 0.01 ml of the acidified PRP were transferred to test tubes held on ice (0-minute)
d. The flask was transferred to a boiling-water bath for 3 minutes, then chilled in an ice-water bath.
e. Step c was repeated (3-minute sample).
f. The samples were assayed for reducing power by the alkaline ferricyanide method standarized with D-ribose.
g. Based on the result (see Table 1), step d was repeated.
h. Step c was repeated (6-minute samples).
i. Step f was repeated.

TABLE 1

Samples	Nanomoles of reducing ribose (av)	Ratio, total ribose/ reducing ribose
0-min	0.42	493
3-min	6.08	34.0
6-min	9.66	21.4

The result (see Table 1) indicated that, assuming the sole mode of hydrolysis had been at the (1-1) glycosidic linkage, the number-average chain length was 21.4 monomeric units, i.e., (ribitol-5-phosphate-3-ribose)

j. 0.102 ml 1N NaOH was added, and the pH was estimated by indicator paper (about pH 6).
k. The neutralized hydrolysate was lyophilized.
l. Bio-Gel P10 (Bio-Rad, Inc.) was equilibrated in 0.1M triethylammonium acetate and poured into a 1.5 cm diameter chromatographic column, giving a gel-bed height of 98 cm.
m. The lyophilized material (step k) was rehydrated with 2.7 ml water, and 0.3 ml of 1M triethylammonium acetate was added. This solution was applied to the column and elution was carried out with collection of 3.5 ml fractions.
n. The elution of ribosyl residues was determined by assay of 0.005-ml samples of each fraction for ribose content by the orcinol reaction with D-ribose as standard.
o. Fractions were combined into 3 pools, L, M, and S as indicated in Table 2, and the pools were assayed for total ribose and reducing ribose:

TABLE 2

Pool	Fractions contained	Total ribose, micromoles	Ratio, total ribose/ reducing ribose	Est. Mn*	Range of Ve/Vo of fraction
L	15–18	577	31.2	11,000	≦1.08
M	19–23	744	18.6	6800	1.09–1.38
S	24–34	1180	9.1	3400	1.39–1.99

*on the assumption that the sole hydrolsis was glycosidic.

p. The pools were lyophilized, re-hydrated with 10 ml water, re-lyophilized, re-hydrated with 1.5 ml water. 1.2 ml of the last solutions were transferred

to microcentrifuge tubes and lyophilized in preparation for the conjugation reactions.

Conjugation of $CRM_{197}$to Reducing Fragments of PRP a. To the microcentrifuge tubes containing lyophilized fragments, L, M, and S and an empty tube (C or control) were added potassium phosphate buffer pH 8, 2.7 milligrams CRM_{197}, and 4 milligrams sodium cyanoborohydride, such that the final volume was 0.2 ml and the phosphate buffer was at 0.2M.

b. The tubes were incubated at 37° C. with daily mixing.

c. After 18 days the tubes were centrifuged 2 minutes at 7000 G.

d. After determination that the majority of protein was in the precipitates, the precipitates were washed four times with ≦1 ml water.

e. The washed precipitates were made 8M in urea and warmed to 50° C., dialyzed against saline overnight at 4° C., and centrifuged. The supernates were separated and made 95% saturated in ammonium sulfate, held overnight at 4°, and centrifuged. The resulting precipitates were washed 3 times with 0.4 ml of 95% saturated ammonium sulfate, and suspended with 1 ml water. These colloidal suspensions were labeled CRM_{197}-PRP-L, -M, -S, and CRM_{197}-C, respectively.

f. The preparations were assayed for protein by means of the Folin phenol reaction with bovine albumin as standard and for ribosyl residues with the orcinol reaction and D-ribose as standard. The results are given in Table 4. The preparations were assayed for PRP antigenic activity by their ability (at concentrations of 50 micrograms protein/ml) to inhibit the binding of labeled native PRP to human anti-PRP antibody (Table 3).

TABLE 3

Preparation tested	% Antigen bound	antigenic activity, ng PRP equivalence/μg protein
none	28.1	—
> native PRP, 0.5 ng/ml	6.7	—
> native PRP, 5 ng/ml	0.94	—
CRM_{197} - C	34.3	0.0
CRM_{197} - PRP-S	2.0	0.1
CRM_{197} - PRP-M	2.5	0.08
CRM_{197} - PRP-L	3.9	0.006

Thus, all the tested conjugates of CRM_{197} with PRP fragments were antigenically active, while the control preparation in which CRM_{197} was exposed to cyanoborohydride in the absence of PRP fragments was inactive as expected.

The preparations were assayed for immunogenicity in rabbits in comparison with high molecular weight purified PRP, and the results are given in Table 4. Rabbits given the PRP control or the CRM_{197} -C control made barely detectable increases in anti-PRP antibody. Rabbits given any of the three CRM_{197} -PRP conjugates made progressive increases after each injection; the titers after the third injection were 1000-fold greater than prior to immunization. In an experiment not illustrated a simple mixture of CRM_{197} and PRP fragment preparation L was assayed in rabbits and found not to elicit anti-PRP antibody.

TABLE 4

ANTI-PRP ANTIBODY RESPONSE TO CONJUGATED AND CONTROL VACCINES OF WEANLING RABBITS PRIMED WITH ORDINARY DIPHTHERIA TOXOID*

	Rabbit Vaccine**	Pentose/protein ratio	Anti-PRP Antibody, ng/ml, at age in weeks 7***	8***	9***	10
1	PRP(MW 10^5)		<10	12	28	40
2	"		<10	<10	27	26
3	CRM_{197}-C (control)	—	35	25	31	36
4	"		16	34	40	48
5	CRM_{197}-PRP-S	0.015	19	980	26,000	49,000
6	"		<10	84	23,000	31,000
7	CRM_{197}-PRP-M	0.0069	<10	37	2,500	11,000
8	"		23	11,000	49,000	150,000
9	CRM_{197}-PRP-L	0.0020	14	73	3,700	26,000
10	"		<10	340	9,800	76,000

*The rabbits were New Zealand Whites obtained from Dutchland Farms immediately after weaning. At six weeks of age each was injected subcutaneously (s.c.) with 40 Lf of diphtheria toxoid (Massachusetts Dept. of Public Health) contained in 0.5 ml of 0.0125 M aluminum phosphate pH 6 (alum).

**The PRP vaccine was 30 μg PRP lot 17 contained in 0.1 ml saline. The other vaccines were 25 μg protein contained in 0.5 ml alumn.

***Injections of the indicated vaccine were given (s.c.) immediately after bleeding. There were two rabbits per vaccine. Listed are individual titers, determined by radio-antigen binding with ^3H-labeled native PRP.

The protective potential of the anti-PRP antibodies induced by the conjugates was evaluated by testing the bactericidal activity of the rabbit sera of Table 4. The bactericidal titers were determined against *H. influenzae* b strain Eag by the methods of Anderson et al, Journal of Clinical Investigation, Volume 65, pages 885-891 (1980). Table 5 shows that before vaccination the sera were unable to kill the bacteria (reciprocal titers <2). After three injections the reciprocal titers of the rabbits receiving the CRM_{197}- PRP conjugates had risen to 16 or greater while titers of the rabbits receiving the CRM 197 control remained at <2.

TABLE 5

Bacterial Titers Against *H. influenzae* b Strain Eag of Sera of Weanling Rabbits Vaccinated With CRM_{197} of Its Conjugates With Oligosaccharides S, M, and L of PRP*

Rabbit	Vaccine given	Reciprocal serum dilution for > 90% Killing: Pre-vaccination	After 3 injections
3	CRM_{197} control	<2	<2
4	CRM_{197} control	<2	<2
5	CRM_{197}-PRP-S	<2	128
6	CRM_{197}-PRP-S	<2	≧256
7	CRM_{197}-PRP-M	<2	16
8	CRM_{197}-PRP-M	<2	64
9	CRM_{197}-PRP-L	<2	64
10	CRM_{197}-PRP-L	<2	32

*Same vaccinations as described in Table 4.

6. EXAMPLE: VARIATION OF PRP FRAGMENT RATIO TO CRM_{197}

In this example, the ratio of PRP fragment S to CRM_{197} was varied and the conservation of antigenic activity of the CRM_{197} component was examined in addition to the PRP component.

Preparation of CRM_{197}-PRP-S#2, A and B.

a. To microcentrifuge tubes A and B were added 0.15 ml each of the solution of fragments S described above, i.e., steps o and p. The solutions were lyophilized.

b. Tube A received 0.015 ml 2M potassium phosphate buffer pH 8, 0.1 ml of CBM_{197} 5 mg/ml in 0.01M

sodium phosphate buffer pH 7, and 0.015 ml of sodium cyanoborohydride 200 mg/ml.

c. Tube B received 0.002 ml of the pH 8 buffer and 0.1 ml of the CRM_{197} solution. The resulting solution was lyophilized. The solids were suspended with 0.015 ml water, and 0.002 ml of the pH 8 buffer were added.

d. Tubes A and B were incubated at 37° C. for 13 days. To tube B an additional 0.002 ml of cyanoborohydride was added. Both tubes were incubated at 37° C. for an additional 3 days. (Note that due to the reduced reaction volume, the concentrations of reactants in B were higher than A.)

e. To A was added 0.06 ml water and 0.8 ml saturated ammonium sulfate (SAS). To B was added 0.175 ml water and 0.8 ml SAS.

f. The tubes were incubated 1 hour at 0° C. and centrifuged 20 minutes at 8000 G. The supernates were removed.

g. The precipitates were washed by suspension in 1 ml of 80% SAS, centrifugation at 8000 G 20 minutes, and removal of the supernates.

h. The precipitates were suspended with 0.1 ml water, and 0.4 ml SAS was added.

i. Same as step f.

j. Same as step g.

k. The precipitate in B was dissolved with 0.084 ml 9.5M urea (estimated final concentration 8M); 0.1 ml water and 0.8 ml SAS were added, and the precipitate was isolated as in step f. This precipitate was washed as in step g.

l. The precipitates in A and B were suspended with 0.2 ml water. The suspensions were separated into soluble (s) and insoluble (i) fractions by centrifugation 30 minutes at 8000 G, and the s fractions (supernates) were made 0.01M sodium phosphate buffer pH and reserved.

m. The i fractions (precipitates) were rendered more soluble as follows: they were made 8M in urea, which was then gradually removed by dialysis against 0.01M sodium phosphate buffer pH 7. The resulting solutions were recombined with the respective s fractions.

n. Preparations A and B were tested for protein content with the Folin phenol reagent and for PRP antigenic activity by the assay described above. Both had PRP activity; B exceeded A by about 13-fold, as shown below:

Preparation	ng PRP equivalence/μg protein
CRM_{197}-PRP-S#2,A	0.038
CRM_{197}-PRP-S#2,B	0.50

o. Preparations A and B were tested for CRM antigenicity (activity as diphtheria toxoid (DT)) by inhibition of the binding of antibody to a sample of purified DT furnished by the Massachusetts Department of Public Health. Both had activity roughly equal to the DT on a weight basis; B exceeded A by about 4-fold, as shown below.

Inhibitor tested	Antibody bound, A_{400}	μg DT equivalence per μg protein
None	2.43	
DT, 0.5 μg/ml	2.56	
DT, 5 μg/ml	1.93	
DT, 50 μg/ml	0.96	
CRM_{197}-PRP-S#2,A,50 μg/ml	1.25	0.52
CRM_{197}-PRP-S#2,B 5 μg/ml	1.67	2.0

p. Preparations A and B were suspended in alum at 16 ug protein 1 ml, and three 0.5 ml injections were given to rabbits in the protocol described in Table 4 (except the animals were 8 weeks old at the onset and not primed by previous injections of diphtheria toxoid). The sera were tested for antibodies in the binding assay described in step o. Both A and B elicited antibodies to DT as well as to PRP, as shown in Table 6. Separate control experiments showed that similar rabbits housed in the same quarters did not display such increases in anti-DT antibody values in the absence of being injected with CRM_{197} preparations.

TABLE 6

Rabbit	Injected	Assay for antibody to	Antibody values at age 8 wk	9 wk	10 wk	11 wk
5	A	PRP, ng/ml	47	60	210	13,500
		DT, A_{400}	0.136	0.168	1.28	3.81
6	A	PRP	21	25	19	420
		DT	0.072	0.049	0.262	3.23
7	A	PRP	<20	20	2000	10,500
		DT	0.155	0.134	0.155	0.676
3	B	PRP	<20	27	1600	4900
		DT	0.075	0.061	0.227	2.45
8	B	PRP	23	<20	2900	26,000
		DT	0.065	0.023	0.231	2.07

7. EXAMPLE: CONJUGATION OF VERY SMALL FRAGMENTS OF PRP TO DIPHTHERIA TOXIN, DIPHTHERIA TOXOID AND CRM_{197}

Generation of Very Small Fragments of PRP Containing Reducing End Groups

a. A 12 ml solution of PRP lot 20 was made 0.1M in HCl at 0° C. and sealed in a glass flask (0 minute).

b. The flask was transferred to a boiling-water bath for 4 minutes, then chilled in an ice water bath.

c. A small amount of resulting white colloid was removed by extraction with ether and the resulting clear solution was lyophilized.

d. Bio-Gel P10 (Bio Rad, Inc.) was equilibrated in 0.01M ammonium acetate and poured into a 1.5 cm diameter chromatographic column, giving a gel bed height of 98 cm.

e. The lyophilized material was rehydrated with 1.5 ml water and neutralyzed with NH_4OH. This solution was applied to the column and the elution was carried out.

f. Fragments eluting at Ve/Vo range of 2.0–2.4 were collected and designated fraction vs.

g. Steps a–f were repeated to double the supply of fraction vs.

h. The combined vs fractions were lyophilized, rehydrated to yield 4 ml of a solution containing a total of 47 umoles of reducing sugar activity when assayed by the alkaline ferricyanide method standardized with D-ribose.

Preparation of Conjugates of PRP-vs Fragments to Native Diphtheria Toxin, Native Diphtheria Toxoid and CRM_{197}

The following proteins are used as carriers in the present example:

(1) DTx—purified diphtheria toxin, lot 1, obtained from the Massachusettes Public Health Biologic Laboratories. Partial detoxification is accomplished by the linking to PRPvs. Residual toxicity is removed by formalin treatment in the presence of lysine by the method of Pappenheimer et al., Immunochemistry, 9:891 (1972).

(2) DTd—conventional (formal) toxoid, lot DCP-27, also obtained from the Massachusettes laboratories.

(3) CRM_{197}—antigenically mutated version of the toxin protein, antigenically indistinguishable from toxin but non-toxic.

The conjugation method is as follows:

a. Protein, potassium phosphate buffer (pH 8.0 at 25° C.) and PRPvs were combined in glass centrifuge tubes in the manner set out below.

Solution	Protein	Buffer	PRPvs
(1)	30 mg DTx	0.24 μmol	20 μmol
(2)	30 mg DTd	0.24 μmol	20 μmol
(3)	10 mg CRM_{197}	0.08 μmol	6.7 μmol

b. The solutions were lyophilized, and the lyophiles were dissolved with $NaCNBH_3$ solution, 2% w/v in water as tabulated below.

Solution	2% $NaCNBH_3$
(1)	1.2 ml
(2)	1.2 ml
(3)	0.4 ml

c. The tubes were incubated at 37° C.

d. After 14 days, four volume-equivalents of saturated ammnonium sulfate were added. These suspensions were held 3 hours at 0° C., then centrifuged 20 minutes at 9000 G.

e. The precipitates were washed twice each with 10 ml of neutral 70% saturated ammonium sulfate.

f. The washed precipitates were dissolved with a minimal volume of 9.5M urea and dialyzed against 0.067M sodium phosphate buffer, pH 7.8.

Formalin Treatment of the Conjugates

a. The conjugates were further dialyzed against sodium phosphate buffer which also contained 0.025M lysine. (Small samples were reserved for toxicity testing prior to formalinization).

b. Formalin was added to a final concentration of 0.2% v/v.

c. After 17 days incubation at about 24° C. the solutions were extensively dialyzed against the sodium phosphate buffer.

d. Centrifugation was performed to remove small amounts of insoluble material.

Processing to Achieve Final Container Products

a. Antigen solutions (1)–(3) in isotonic sodium phosphate buffer were passed through 0.22-micron "Millex" filter units (Millipore Corp.) and injected into bottles containing sterile phosphate buffered saline.

b. The preparations were assayed for protein using the Lowry method.

c. Thimerosal was filtered and injected into the solution as 1/100 volume of a freshly made 1% w/v solution. Samples of 10 ml were taken for a sterility test. The bottles were attached to a manually operated sterile single use filling device (Multiple Additive Set, Travenol Laboratories). 2-ml glass vials were filled, stoppered, sealed, and immediately transferred to storage at 4° C.

Assays on Conjugate Preparations

a. Phosphate content of the protein fraction

PRP is composed of the repeating unit ribosyl-ribitol-phosphate. Thus colorimetric assay of phosphate in the fraction precipitable by 5% tricholoracetic acid (TCA) is a sensitive index of the incorporation of PRP fragments into the protein.

Samples containing 100 μg protein were made 5% in TCA in a volume of 3 ml, held 20 minutes on ice, and centrifuged 15 minutes at 4° C. at G. The precipitates were washed with an additional 3 ml of 5% TCA, then with 5 ml ethanol. The washed precipitates were ashed to convert organic phosphate to inorganic phosphate (Pi), and the Pi was quantified by the method of Chen et al., Anal. Chem., 28:1756 (1956). The results were as follows:

Sample	nmol Pi/ μg protein	Implied average no. of PRP repeating units/protein
(1) DTx-PRPvs	0.11	6.8
(2) DTd-PRPvs	0.10	6.2
(3) CRM_{197}-PRPvs	0.10	6.2

b. Electrophoretic Analysis

Samples of the conjugated antigens were analyzed by mercaptoethanol-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (ME-SDS-PAGE) in the same gel alongside the respective starting carrier protein preparations.

DTd-PRPvs, like the DTd, displayed a disperse band at MW 61,000 daltons. In contrast, DTx-PRPvs and CRM_{197}-PRPvs differed greatly from the starting proteins. The protein of these two conjugates collected either at the beginning of or in the stacking gel (4% acrylamide) or at the beginning of the separating get (10% acrylamide). Thus, the conjugates appear to have been converted into macromolecular aggregates, presumably by cross-linking from the formalin treatment. DTd-PRPvs also contains some aggregated material.

c. PRP Antigen Equivalence per Unit Protein

The capacity of the conjugates to bind anti-PRP antibody was determined by the inhibition of the binding of labeled PRP by human anti-PRP antiserum, calibrated with PRP lot 19. (Because protein-bound polymer fragments cannot be assumed to bind to antibody in a weight-equivalent fashion to the high molecular weight polymer, quantitative chemical composition cannot be inferred from these data.)

Sample	% Inhibition of ^3H-PRP bound	ng PRP equivalence/ μg protein
PBS control	(0)	—

-continued

Sample	% Inhibition of ^{3}H-PRP bound	ng PRP equivalence/ μg protein
PRP 19, 0.5 ng/ml	6.7	—
PRP 19, 5 ng/ml	32	—
PRP 19, 50 ng/ml	90	—
DTx-PRPvs, 5 μg protein/ml	24	0.5
DTd-PRPvs, 5 μg protein/ml	48	2.2
CRM_{197}-PRPvs, 5 μg protein/ml	38	1.4

d. Diphtheria Toxoid Antigenic Equivalene Per Unit Protein

Retention of the capacity of the preparations to react with anti-DTd antibody was determined by inhibition of an enzyme-linked immunosorbent assay (ELISA) in which purified DTd is attached to the assay tube (solid phase). Inhibition of antibody binding to the attached DTd is calibrated by the same DTd used in the fluid phase.

Sample	% Inhibition of Antibody Binding	μg DTd equivalence/ ug protein
PBS control	(0)	—
DTd, 5 μg protein/ml	24	—
DTd, 50 μg protein/ml	50	—
DTx-PRPvs, 50 μg protein/ml	46	0.68
DTd-PRPvs, 50 μg protein/ml	58	2.1
CRM_{197}-PRPvs, 50 μg protein/ml	26	0.11

e. Diphtheria Toxic Activity

Samples of the original DTx and the conjugate DTx-PRPvs before and after formalin treatment were titrated for toxic activity by injection into the skin of a non-immune adult rabbit. DTx at doses of 0.002 μg and 0.02 μg produced the expected dermal lesions. DTx-PRPvs prior to formalin treatment produced dose-dependent lesions such that 0.2 μg was approximately equal to 0.002 μg DTx (100-fold reduction in toxicity by the conjugation). After formalin treatment, lesions were not generated by doses as high as 2 μg (at least 1000-fold reduction relative to DTx). Doses up to 2 μg of conjugates DTd-PRPvs and CRM_{197}-PRPvs were tested similarly and generated no lesions.

f. Induction of Anti-PRP Antibody Responses in Weanling Rabbits, Measured by Radioantigen binding

The antigens were mixed with an aluminum phosphate adjuvant (0.0125M Al, pH 6) such that a 0.5 ml dose contained 25 μg protein. Two rabbits (for each antigen) were given three weekly injections beginning at age 7 weeks; the rabbits had been injected with DTd alone at age 5 weeks, for a hypothetical "carrier priming" effect. All the animals (rabbits 1–6) had anti-PRP rises in an anamnestic pattern, with titers of at least 10 μg/ml after the third vaccination. Antigens CRM_{197}-PRPvs and DTd-PRPvs were also tested in pairs of rabbits that had not been "primed" with DTd. These (rabbits 7–10) produced strong anti-PRP reponses similar to those in the "primed" rabbits.

g. Induction of Anti-DTd Antibody Response in Weanling Rabbits, Measured by ELISA

The anti-DTd antibody responses in the same "unprimed" rabbits (7–10) of the preceding subsection are as follows: Rises were roughly 10-fold after the second injection and another 2-to 5-fold after the third.

h. Sterility of the Sample Preparations

The samples were found to be sterile as determined using Fluid Thioglycollate (BBL cat. no. 11260, lot D4D LKL) as the growth medium.

8. EXAMPLE: USE OF PRP FRAGMENTS CONJUGATED TO DIPHTHERIA TOXOID AND CRM_{197} AS VACCINES IN YOUNG HUMANS

Two groups of 8 children in the age range of 1 to 2 years old, (and specifically exempting children receiving routine vaccination with diphtheria toxoid protein at age 18 months) were given primary and secondary vaccinations as follows: Group I received injections of CRM_{197}-PRPvs, preparation as described in the preceding section, (25 μg protein in saline, subcutaneously); Group II received injections of DTd - PRPvs, preparation as described in the preceding section, (25 μg protein in saline, subcutaneously).

In the first visit, pre-vaccination blood specimens were taken; the child was vaccinated, then observed for 20 minutes for any sign of an anaphylactic reaction. In the second visit the procedure of the first visit was repeated. In the third visit, a post-secondary blood specimen was taken. Two of the children, one from each group, after consultation with the parents, were given a third vaccination to try to raise the antibody against PRP to protective levels. The interval between vaccinations was $1 \pm \frac{1}{2}$ month.

Group III consisted of children about 18 months old receiving a vaccine simultaneously with diphtheria toxoid protein in a separate site. This group contained 2 children; one received the CRM_{197}-PRPvs vaccine, the other received the DTd-PRPvs vaccine.

Symptoms were recorded for four successive days, with measurements of temperature, notation of behavioral indications of systemic illness and observations of inflammation at the injection site. These symptoms are summarized in Table 7.

TABLE 7

ADVERSE REACTIONS TO PRP-VS CONJUGATES TO CRM_{197} AND FORMAL DIPHTHERIA TOXOID

Vaccine	Symptom	Injection: Primary	Injection: Secondary	Injection: Tertiary
CRM_{197}-PRPvs	Fever	1/8	0/8	0/1
	Unusual behavior	0/8	0/8	0/1
	Local inflammation	1/9*	2/9	0/1
	Local pain	1/9*	1/9	0/1
DTd-PRPvs	Fever	0/8	0/8	0/1
	Unusual behavior	0/8	0/8	0/1
	Local inflammation	1/9*	0/9	0/1
	Local pain	1/9	1/9	0/1

*Includes one child who received diphtheria toxoid protein simultaneously in a separate site. No local symptoms were found. Systemic symptoms are not noted since these could not be distinguished from an effect of the diphtheria toxoid protein vaccine.

After CRM_{197}-PRPvs vaccination, one child had mild fever (99.8° C.) on the evening of primary vaccination; there was an instance of mild local inflammation once each after a primary, a secondary, and the one tertiary vaccination. After DTd-PRPvs there was an instance of local inflammation after one primary and one secondary vaccination. The administration of the vaccines was otherwise apparently free of adverse reactions.

Serum Antibody Reponses

Antibodies to PRP as well as IgG antibodies to diphtheria toxoid were determined. After vaccination with CRM_{197}PRPvs a consistent anti-PRP response pattern was seen. See Table 8. There was a distinct rise after the primary injection, usually an even larger rise after the secondary injection, and a large rise after the one tertiary. The final titers greatly exceeded those that have been produced by vaccination with PRP alone and greatly exceeded the accepted estimated protective minimal level of 0.15 μg/ml. The enhanced response was particularly evident in the four children under 18 months of age, where the response to PRP alone is generally inadequate for protection, and the geometric mean of the final titers in these four (8.4 μg/ml) is 175 times that found after vaccination of children 12–17 months old with PRP vaccine alone. The child receiving the primary vaccination simultaneously with diphtheria toxoid protein vaccine also had an excellent response.

IgG antibodies to diphtheria toxoid increased in 6 of 8 children (as well as in the 9th, who also received diphtheria toxoid as part of the treatment). The antibody levels often increased so greatly that the dilution of post-vaccination serum used (1/1000) was insufficient to show the full extent of the rise.

After vaccination with DTd-PRPvs anti-PRP responses generally increased after both primary and secondary vaccination. (See Table 9). However, there were two children (12 and 14 month old) in whom no response was detected; and one child did not approach the protective level until given a third injection. The child receiving the primary vaccination simultaneously with diphtheria toxoid protein had an excellent response. Rises in IgG antibody to the diphtheria component were found in all children.

TABLE 8

ANTIBODY RESPONSE TO CRM_{197}-PRPvs

Subject	Age at primary vaccination	Serum sample	Serum antibody, μg/ml anti-PRP	IgG anti-DTd
1	12 mo	pre-vac	2.0	1.1
		post-1	4.5	>10
		post-2	18	>10
2	13 mo	pre-vac	<0.006	0.38
		post-1	0.040	1.7
		post-2	0.35	2.2
		post-3	4.8	1.9
3	14 mo	pre-vac	<0.020	4.5
		post-1	0.12	3.3
		post-2	2.0	4.3
4	16 mo	pre-vac	0.025	0.06
		post-1	0.92	5.7
		post-2	29	9.1
5	27 mo	pre-vac	0.025	3.0
		post-1	10	>10
		post-2	58	>10
6	29 mo	pre-vac	0.13	6.1
		post-1	22	6.9
		post-2	180	7.4
7	30 mo	pre-vac	2.2	6.5
		post-1	28	>10
		post-2	50	>10
8	30 mo	pre-vac	1.3	4.8
		post-1	6.5	>10
		post-2	78	>10
9	18 mo*	pre-vac	0.34	3.1
		post-1	1.4	>10
		post-2	8.2	>10

*First injection of CRM_{197}-PRPvs given simultaneously with diphtheria toxoid protein vaccine in a separate site

TABLE 9

ANTIBODY RESPONSE TO DTd-PRPvs

Subject	Age at primary vaccination	Serum sample	Serum antibody, μg/ml anti-PRP	IgG anti-DTd
1	12 mo	pre-vac	<0.020	0.060
		post-1	<0.020	10
		post-2	<0.020	10
2	12 mo	pre-vac	0.055	0.03
		post-1	0.080	3.1
		post-2	1.8	10
3	13 mo	pre-vac	<0.006	1.1
		post-1	<0.006	10
		post-2	0.023	10
		post-3	0.120	10
4	14 mo	pre-vac	<0.020	3.0
		post-1	<0.020	5.1
		post-2	<0.020	3.8
5	19 mo	pre-vac	0.060	8.0
		post-1	0.12	10
		post-2	0.76	10
6	26 mo	pre-vac	<0.020	6.9
		post-1	0.060	10
		post-2	0.94	10
7	27 mo	pre-vac	1.4	6.1
		post-1	7.4	10
		post-2	21	10
8	28 mo	pre-vac	<0.020	8.7
		post-1	0.63	10
		post-2	8.0	10
9	18 mo*	pre-vac	1.9	0.11
		post-1	2.9	10
		post-2	11	10

*First injection of DTd-PRPvs given simultaneously with diphtheria toxoid protein vaccine in a separate site

This example shows that injections of conjugates of the *H. influenzae* b capsular polymer fragment to diphtheria toxoid and CRM_{197} is apparently harmless. CRM_{197}-PRPvs vaccination gave a clear indication of an enhancement of the anti-PRP response by the carrier effect —appreciated not only by the high titers but by the rises after secondary vaccination.

DTd-PRPvs had a less impressive enhancement. A likely explanation is that while CRM_{197}-PRPvs is a multimolecular aggregate, DTd-PRPvs is present mainly in unimolecular form similar to the original toxoid.

9. EXAMPLE: CONJUGATION OF CAPSULAR POLYMER FRAGMENTS OF STREPTOCOCCUS PNEUMONIAE TO CRM_{197}

Several other bacteria resemble *H. influenzae* b in that they cause sepsis and meningitis, particularly in infants; they have polymer capsules, antibodies to which are protective; and their capsular polymers are immunogenic in mature humans but not in infants. An important example is *Streptococcus pneumoniae* (Sp) serotype 6. It causes not only the life-threatening infections mentioned above but also is a highly prevalent cause of otitis media in children. (Gray et al, Journal of Infectious Diseases, Volume 142, pages 923–33, 1980).

The approach described for PRP is also applicable to any capsular polymer in which reducing groups can be generated by selective hydrolysis with retention of antigenic specificity. In the following non-limiting example, capsular polymer fragments were made from the Sp. 6 capsular polymer by selective acid hydrolysis and were conjugated to CRM_{197}. The product retained antigenic specificity for both the Sp capsular polymer and the CRM_{197} component.

Generation of Reducing Fragments From Capsular Polymers (CP)

1. A sample of the CP of Sp. 6 (Danish type 6A, Eli Lilly Co.) was assayed for total hexose by the phenol-sulfuric acid method standardized with D-glucose and for reducing activity by the alkaline ferricyamide method also standardized with D-glucose.

2. A Pyrex tube received 3.3 mg Sp. 6 CP dissolved with 0.66 ml water. The sample was chilled to 0° C., 0.073 ml of 0.1N HCl were added, and the tube was sealed.

3. The tube was immersed 10 minutes in a boiling water bath, then rechilled to 0° C. A small sample was assayed for reducing activity as described in step 1:

CP	Time heated at 100° C.	Total hexose/ reducing hexose
Sp. 6	0 minutes	>350
	10 minutes	6.5

4. The hydrolyzed preparation (minus the 2% used for assay) was lyophilized. The dried material was dissolved with 0.1 ml water, transferred to microcentrifuge tube, and lyophilized again.

Conjugation to CRM_{197} 1. To the re-dried hydrolysate was added 0.004 ml of 2 M potassium phosphate buffer pH 8 and 1 mg of CRM_{197} dissolved in 0.2 ml of 0.01M sodium phosphate buffer pH 7. The resulting mixture was lyophilized and resuspended with 0.05 ml water (estimated total volume 0.063 ml).

2. To the tube was added 0.007 ml of sodium cyanoborohydride at 200 mg/ml, and the preparation was incubated 18 days at 37° C.

3. 0.6 ml 80% saturated ammonium sulfate (SAS) was added.

4. The tube was incubated 1 hour at 0° C. and centrifuged 15 minutes at 8000 G; the supernate was removed.

5. The precipitate was washed by suspension in 0.6 ml of 80% SAS buffered at pH 8 with 0.01M sodium phosphate, followed by centrifugation 15 minutes at 8000 G.

6. The precipitate was suspended with 0.02 ml of 0.5M Na_2HPO_4 and 0.2 ml 9.5M urea.

7. 1 ml SAS was added, the precipitate was isolated as in step 4 and suspended in urea at about 8M as in step 6.

8. The suspension was centrifuged 15 minutes at 8000 G.

9. The supernate was separated and dialyzed against 0.01M sodium phosphate buffer pH 7 at 4° C.

10. The dialyzed preparations, designated CRM_{197}-Sp. 6 was assayed for the following:

protein by the Folin phenol reaction;

Sp antigenicity by inhibition of binding of antibody to radiolabeled Sp CP (as described for PRP in Table 3);

CRM_{197} antigenicity by the inhibition of antibody binding to diphtheria toxoid (DT) (as described in step o of the description of CRM_{197}-PRP-S#2); and

anti-CP immunogenicity by inhibition of the binding of antibody to diphteria toxiod (DT) (as described in step p of the description of CRM_{197}-PRP-S#)

Preparation	ng CP equivalance/ μg Protein	μg DT equivilance/ μg protein
CRM_{197} Sp. 6	0.4	0.36

TABLE 10

ANTI-CP IMMUNOGENIC RESPONSE OF WEANLING RABBITS WITH CONTROLS AND CONJUGATES OF STREPTOCOCCUS PNEUMONIAE SEROTYPE 6 FRAGMENTS OF CRM_{197}

Rabbit	Vaccinated With*	Percent ^{14}C-CP Bound in Samples at age** 6 wk	8 wk	10 wk	11 wk
1	Sp 6 CP, 25 μg	6	6	7	7
2	"	6	13	13	11
3	Sp 6 bacteria 25 μg	4	10	12	16
4	"	8	12	22	21
5	CRM_{197} Sp 6, 25 μg	4	6	30	49
6	"	4	8	30	54

*Injected subcutaneously just prior to taking serum samples. Serum samples were taken at age 6, 8 and 10 weeks.
**25 ul serum incubated with 2 nCi ^{14}C-labelled CP.

I claim:

1. immunogenic conjugate comprising the reductve amination product of an immunogenic capsular polymer fragment having a chain length of from about 10 to about 30 monomeric units and a reducing end, which fragment is derived from the capsular polymer of a *Streptococcus pneumoniae* or *Haemophilus influenzae bacterium,* and a bacterial toxin or toxoid.

2. The immunogenic conjugate of claim 1, wherein the capsular polymer is immunogenic in mature humans and less immunogenic in infant humans.

3. The immunogenic conjugate of claim 1, wherein the reductive amination is performed in the persence of cyanoborohydride anions.

4. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is diphtheria toxin or toxoid.

5. The immunogenic conjugate of claim 4, wherein the toxoid is CRM_{197}.

6. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is tetanus toxin or toxoid.

7. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is a pseudomonas toxin or toxoid.

8. The immunogenic conjugate of claim 1, wherein the toxin or toxiod is a staphylococcus toxin or toxoid.

9. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is a streptococcus toxin or toxoid.

10. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is pertussis toxin or toxoid.

11. The immunogenic conjugate of claim 1, wherein the toxin or toxoid is *Escherichia coli* toxin or toxoid.

12. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Haemophilus influenzae* type b.

13. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 3.

14. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 6.

15. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 12.

16. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 14.

17. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 19.

18. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 23.

19. The immunogenic conjugate of claim 1, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 51.

20. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Haemophilis influenzae* type b.

21. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 6.

22. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 14.

23. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 19.

24. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 23.

25. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by oxidative cleavage.

26. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by periodate.

27. The immunogenic conjugate of claim 1, wherein the fragment is derived from the capsular polymer by hydrolysis of a glycosidic linkage.

28. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished enzymatically.

29. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished chemically.

30. The immunogenic conjugate of claim 27, wherein the hydrolysis is accomplished by acid.

31. The immunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of $\leqq$1.08.

32. The immunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.09–1.38.

33. The imrhunogenic conjugate of claim 12, wherein the fragment elutes on a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.39–1.99.

34. An immunogenic conjugate comprising a formalin treated reductive amination product of an immunogenic capsular polymer fragment having a chain length of from about 10 to about 30 monomeric units and a reducing end, which fragment is derived from the capsular polymer of a *Streptococcus pneumoniae* or *Haemophilus influenzae* bacterium, and a bacterial toxin or toxoid.

35. The immunogenic conjugate of claim 34, wherein the bacterial toxoid is diptheria toxoid.

36. The immunogenic conjugate of claim 35, wherein the Toxoid is CRM_{197}.

37. The immunogenic conjugate of claim 34, wherein the bacterial toxin or toxoid is tetanus toxin or toxoid.

38. An immunogenic conjugate of (1) a PRP polysaccharide fragment having reducing terminal groups derived from the capsular polysaccharide of *Haemophilus influenzae* type b by selective acidic hydrolysis of a portion of the ribosyl ribitol linkages therein and (2) the diphtheria toxin protein CRM_{197}.

39. The conjugate of claim 38 prepared by the reductive amination of the PRP fragment and protein.

40. The conjugate of claim 38 prepared by reductive amination in the presence of cyanoborohydride anions.

41. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of $\leqq$1.08.

42. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.09–1.38.

43. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 1.39–1.99.

44. The conjugate of claim 38 wherein said PRP fragment elutes from a column of Bio-Gel P-10 at a Ve/Vo ratio of 2.0–2.4.

45. A vaccine that elicits effective levels of anti-capsular polymer antibodies in humans, comprising: the immunogenic conjugate of claim 1.

46. A method for actively immunizing humans against a bacterial pathogen having a capsular polymer, comprising: administering an effective amount of the vaccine of claim 45.

47. A vaccine that elicits effective levels of anti-PRP antibody formations in young warm-blooded mammals comprising an immunogenic amount of the conjugate of claim 41 and a pharmaceutically acceptable carrier.

48. A vaccine that elicits effective levels anti-PRP antibody formations in young warm-blooded mammals comprising an immunogenic amount of the conjugate of claim 42 and a pharmaceutically acceptable carrier.

49. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptoccoccus pneumoniae* serotype 3.

50. The immunogenic conjugate of claim 5, wherein the bacterial pathogen is *Streptococcus pneumoniae* serotype 51.

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